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Seroprevalence of Contagious Bovine Pleuropneumonia in Eastern Sudan with Development of a Rapid Test for Serodiagnosis

معدل الانتشار المصلي لمرض الالتهاب الرئوي البلوري الساري في الأبقار- شرق السودان مع إنتاج اختبار سريع للتشخيص المصلي

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بسم الله الرحمن الرحيم

قال تعالي:

{وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ ۖ وَسَتُرَدُّونَ إِلَىٰ عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُمْ بِمَا كُنْتُمْ تَعْمَلُونَ}

سورة التوبة- الايه (105)

PREFACE

This study was carried out at Mycoplasma Department, Central Veterinary Research Laboratory (CVRL) -Sudan, under the supervision of Prof. Galal Eldin El Azhari Mohammed and co-supervision of Dr. Faisal Mohammed Hamid.

DEDICATION

To soul of my mother and father

To soul of my second mother Amal Mustafa Mohammed Ali

To my brothers and sisters

To my small family

(My husband Omer and My sons; Mohammed, Abubaker and Yousif)

To all my friends

with best wishes

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ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is one of the serious threats to the livestock in Sudan. For effective control of CBPP, early diagnosis is the cornerstone. Culture method is considered laborious, expensive and time consuming. The aim of this study was to estimate the seroprevalence and geographical distribution of Contagious Bovine Pleuropneumonia (CBPP) in Eastern states of Sudan, and to identify the highly risk areas within its localities. The objective also included isolation and full identification of new strain of *Mycoplasma mycoides* subsp *mycoides* using conventional, molecular and bioinformatic techniques. Development of stained antigen for sero- diagnosis of CBPP, from local strain besides T1/44 reference strain was one of the main targets of this study. A total of 1960 serum samples were collected randomly from cattle in Al Gedaref, Red Sea and Kassala states. The samples were tested using competitive ELISA (c. ELISA). The highest seroprevalence was observed in Al Gedaref state (12%), followed by Kassala (6.9%) and Red Sea state (4.1%). In Al Gedaref state; Al Galabat Eastern and Al gurisha localities had scored the highest seroprevalence (17.1%). In Kassala state the highest seroprevalence was observed in Naher Atbara locality (17.1%). In Red Sea state the highest seroprevalence was observed in Sawakin locality (11.4%). *Mycoplasma mycoides* subsp *mycoides* (*Mmm*) strain was isolated from clinically diseased animal and characterized using conventional and molecular techniques. Using bioinformatics tools the isolated RH strain was confirmed to be *Mmm* and 100% similar to certain strains in gene-bank (PG1and Vmm).

Two types of stained antigen were developed for using in sero-diagnosis of CBPP. First antigen was produced from T1/44 reference

strain (SAT_1) and the second one was produced from (RH) local strain (SAT_2). The antigens were evaluated using standard positive and negative reference serums. Competitive ELISA -as a golden test- was used to estimate their sensitivity and specificity beside Latex agglutination test (LAT). The antigens (SAT_1 and SAT_2) were highly sensitive and specific (100%) when tested against 15 positive serum samples from infected herd (confirmed by isolation of *Mmm*). Statistical analysis using Pear-son Chi-square test and Kappa test revealed that the sensitivity of the developed stained antigen (compared with c.ELISA) from T1/44 strain (SAT_1) was 100% in comparison of 92% and 95% of SAT_2 (RH local strain) and LAT, respectively. The specificity of the developed SAT_2 antigen was (70%) when compared with c. ELISA, which was higher than SAT_1 (31%) and LAT (59.6%). From these results it could be concluded that SAT_2 - which was produced from local strain proved to be more specific and highly sensitive with low cost and easy application in the field.

الخلاصة

مرض ذات الرئة البلوري الساري في الأبقار (أبوقنيت) من الأمراض الخطرة والمؤثرة علي القطعان في السودان. التشخيص المبكر يعتبر من أهم الوسائل للتحكم في المرض. التزريع للعامل المسبب للمرض من وسائل التشخيص ولكنه يحتاج لوقت وجهد. الهدف من الدراسة وجه لتحديد الوضع الراهن للمرض وتوزيعه جغرافياً في الولايات الشرقية من السودان وتوضيح أكثر المناطق إصابة داخل محلياتها. كذلك من أهداف الدراسة عزل المايكوبلازما مايكويدس مايكويدس باستخدام الطرق التقليدية وتقنية الأحياء الجزيئية وتعريفها باستخدام تقنية المعلوماتية الحيوية. إنتاج مستضد مصبوغ للتشخيص السيرولوجي للمرض باستخدام عتره محلية بجانب العتره المرجعية (T1/44) واحد من أهم أهداف الدراسة. وفي التقصي الحقلي السيرولوجي للمرض، تم جمع 1960 عينة من السيرم عشوائياً من ولايات شرق السودان شاملة ولاية القضارف ، البحر الأحمر وكسلا . تم فحص المصل باستخدام اختبار الإليزا التنافسية للكشف عن وجود أجسام مضادة لمسبب المرض. أثبتت نتائج المسح المصلي أن أعلي نسبة إصابة بالمرض كانت في ولاية القضارف بنسبه 12.2%، تليها ولاية كسلا بنسبة 6.7%، وفي ولاية البحر الأحمر كانت نسبة الإصابة4.1 %. في ولاية القضارف سجلت محلية القلابات الشرقية ومحلية القريشة أعلي نسبة إصابة (17.1%) ، أما في ولاية كسلا سجلت محلية نهر عطبرة أعلي نسبة إصابة (17.1%) . في ولاية البحر الأحمر سجلت محلية سواكن أعلي نسبة إصابة (11.4%). تم عزل المايكوبلازما المسببة للمرض من حيوان مصاب، وأُجريت الاختبارات التقليدية والحديثة للتأكد منها، بالإضافة لتطبيق المعلوماتية الحيوية لمقارنتها حيث أثبتت أنها مايكوبلازما مايكويدس مايكويدس و تطابق بعض المعزولات في بنك الجينات بنسبه 100% وهما PG1 و Vmm. تم إنتاج مستضدان للتشخيص السيرولوجي لمرض ابوقنيت أحدهما يحتوي علي العتره الأجنبية المعروفة (T1/44) ويسمي (SAT_1) والأخر علي العتره المحلية ويسمي (SAT_2). الأنتجينات المنتجة تم تقييمهما باستخدام أمصال قياسيه مرجعية واستخدمت الإليزا التنافسية لتحديد مدي حساسيتهما وخصوصيتها لتشخيص المرض بجانب إختباراللاتكس التلازني . الاختباراتSAT_1وSAT_2 ذوي حساسية وخصوصية عالية بنسبه 100 %،عندما فحصت بهما 15 عينة سيرم موجبة من قطيع مصاب بأبوقنيت (تم التأكد منها بعزل

العامل المسبب). أوضحت نتائج التحليل الإحصائي أن حساسية المستضد المنتج من العتره الأجنبية كانت نسبتها 100 % بالمقارنة مع 92% و 95% للمستضد المنتج من عتره محلية وإختبار اللاتكس التلازني المستورد علي التوالي. بالنسبة لتطابق المستضدات أعطي المستضد المحتوي علي العتره المحلية أعلي نسبه 70%، يليه إختبار اللاتكس التلازني المستورد 59.6% و 31% بالنسبة للمستضد المحتوي علي العتره الأجنبية. خلصت هذه الدراسه بأن المستضد المنتج من العتره المحلية أكثر تطابقاً وذو حساسية عالية، وكذلك سهل الاستخدام ولا يحتاج لأدوات خاصة معقدة.

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INTRODUCTION

Contagious Bovine Pleuropneumonia (CBPP) is one of the most threatening transboundary and economically important cattle diseases in Africa. The disease is caused by *Mycoplasma mycoides* subsp *mycoides* (*Mmm*) (Billy *et al*., 2015; OIE, 2018). It is an acute, subacute or chronic disease that affects only cattle and occasionally water buffalo (*Bubalus bubalis*) (Santini *et al*., 1992).

Due to high economic losses caused by CBPP in endemic regions, The World Organization for Animal Health (OIE) declared that CBPP as one of the most serious contagious animal disease and listed it in the group of notifiable animal diseases of high socio-economic impact and is regarded as one of the major transboundary animal diseases (TADs) (FAO, 2002; Wade *et al.*, 2015). Therefore, control of the disease is a priority in endemic areas. The major obstacles to eradication of CBPP is the difficulties in controlling cattle movement, applying quarantine and slaughter policies (OIE, 2018). Other difficulties arise due to expensive kits of a field test for diagnosis and the relatively short duration of post-vaccinal immunity. CBPP is endemic to part of Africa, although it has been eradicated in other parts of the world through the application of restrictions to the movement of cattle, as well as test and slaughter policies combined with compensation for livestock owners during the mid 20th century **(**Sacchini *et al.*, 2012).

In Africa control of the disease is based on vaccination campaigns using attenuated strains such as T1/44 or T1/sr. Although the use of antibiotics is theoretically prohibited, they are widely applied in the field (OIE, 2018).

The main problem in eradication is the frequent occurrence of sub-acute or asymptomatic infections and the persistence of chronic carriers after the clinical phase (OIE, 2018).

Laboratory diagnosis of CBPP is based on indirect tests such as the complement fixation test (CFT), ELISA, and immunoblotting (IB), as well as direct methods such as culture and polymerase chain reaction (OIE 2018). Even if not routinely used to diagnose CBPP, immunohistochemical (IHC) technique remained for long time among the diagnostic tests proposed to demonstrate the presence of *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) or its antigens (OIE 2008).

Epidemiological studies of the disease are important to carry out an effective control of CBPP through strategic vaccination.

Development of rapid test at (CVRL-Sudan); due to expensive and slow flow of diagnostic kits was one of the main causes to carry out this research work.

The overall objectives of this study were seroprevalence study to assess the distribution of CBPP in Eastern states of Sudan and to contribute in developing rapid diagnostic kits for CBPP diagnosis.

Specific objectives were:

1- Estimation of CBPP seroprevalence in Eastern states of Sudan including: Al Gedaref, Red Sea and Kassala states using Competitive ELISA.

2- Isolation and full identification of recent *Mycoplasma mycoides* subsp. *mycoides* candidate, to be used for local diagnostic antigen.

3- Appling the Bioinformatics analysis to identify the similarity and differences between the isolated strain and other strains in gene-bank.

4- Development of stained agglutination antigen from reference and local strains with comparative studies using c.ELISA- as a golden test -beside assessment of Latex Agglutination Test, and apply of statistical analysis to assess the sensitivity, specificity and other parameters on the developed antigens.

CHAPTER ONE

LITRATURE REVIEW

1.1. History and distribution of CBPP disease

Contagious Bovine Pleuropneumonia (CBPP) was first reported in Germany in 1693 (Anon, 1976) .The disease rapidly spread over whole Europe, after that it was carried to South Africa, Australia, and United states.

The spread of the disease was greatly helped by the development of the international trade in the middle of nineteenth century especially to England and Scandinavian countries.

By 1803, the disease was reported in the United State of America (Jasper, 1967). Between the 80's and early the 90's, CBPP outbreaks have occurred in Southern Europe, involving Portugal, Spain, France, and Italy (Done *et al*., 1995). The disease was eradicated from Europe by 1999, through a policy of 'stamping out' of infected herds, animal movement restriction, and trace back. Surveillance was based on serological testing and post mortem inspection of slaughtered animals (Regalla *et al.*, 1996, Giovannini *et al.*, 2000).

In Africa the disease was introduced to South Africa due to importation of cattle from Netherland, and thus the disease was spread to other countries (Provost *et al*., 1987).The disease was given top priority by Pan African Control of Epizootics (PACE) after the successful eradication of Rinderpest.

By the mid-20th century its incidence began to decline in Africa by the 1970s. However, because of the economic and financial difficulties that affected the ability of governments to adequately fund veterinary services, the disease came back in the late 1980s and early 1990s (Tambi *et al.,* 2006; Rovid, 2008).

In 2011, CBPP was reported to the AU-IBAR (African Union Inter-African Bureau for Animal Resources) from 18 African countries; spreading across the west, central, east and southern Africa regions. During the reporting period, 304

epidemiological units were affected by CBPP across Africa involving 16,836 cases and 3007 deaths, with an estimated case fatality rate of 17.9%. The highest number of CBPP outbreaks was reported in Ghana (75) followed by Central African Republic (43) and Ethiopia (29). Gabon and DRC have been reporting the disease for the first time in 2010.While the rest listed affected countries reporting the disease over the past four years (Table1.1).

Table 1.1: Countries in Africa reporting CBPP to the AU-IBAR

Country	Outbreaks	Cases	Deaths	Slaughtered	Destroyed
Burkina Faso	4	203	45	0	0
Cameroon	8	384	16	41	0
Central African Republic	43	3674	1270	0	0
Chad	17	342	200	37	18
Congo, DRC	15	8277	458	1361	0
Cote d'Ivoire	18	595	215	13	7
Ethiopia	29	457	112	12	0
Gabon	3				
Ghana	75	127	1	115	0
Mali	4	204	82	119	0
Niger	6	41	10	0	0
Nigeria	22	489	96	221	9
Somalia	12	69	16	0	0
Sudan	2	202	92	108	0
Tanzania	8	399	177	0	0
Togo	9	13	3	1	0
Uganda	22	1330	193	67	0
Zambia	7	30	21	0	1
Total	304	16,836	3007	2095	35

Source: (AU-IBAR, 2011)

In Sudan CBPP is considered the most serious disease of economic importance, which adversely affects foreign trade (Abdulla 1975, Shallali *et al*.,1998). CBPP has been enzootic since the beginning of this century. It is considered one of the most serious diseases of cattle in Sudan, leading to economical losses in forms of debilitation and death of sick animals and adversely affecting foreign trade (Abdulla, 1975; Mohamed Babiker, 2005).The disease was first observed in 1875 in Darfur Province and later spread to Khartoum Province where it caused great losses among cattle (Anon, 1925). The disease disappeared during the Mahdi wars in 1889, and it reappeared again in Kordofan Province in 1912. From there the disease spread quickly southwards and eastwards of the Province. In 1913 the disease was reported in Nuba Mountains, the White Nile, Blue Nile Provinces. In 1914 the disease reached Khartoum Province and then spread to Berber Province in 1913 and Kassala Province in 1917 (Anon, 1925, Mohamed Babiker, 2005). The disease is frequently reported from Eastern states near Ethiopian border in Doka and Al Galabat in Al Gedarif State (Anon, 1925).Al Managel (Gezira State), Al Dinder and Singa (Sinnar State), Al Rossires and Al Angasana (Blue Nile State), Algabalin and Kosti (White Nile State) are endemic areas in the Central States (Anon, 1925). In the northern River Nile and Northern state and Eastern states (Kassala and Red Sea) the disease has not been reported for more than 20 years (Shalalli *et al*., 2008). In Darfour Province- Omdafoug locality, Tassin (2018) found the seroprevalence of the disease was 9.5%. In Khartoum Province many recent researches had done to estimate CBPP seroprevalence. It was 42.2% by Isam (2005), 57% by Amira (2009), 17.19% by Ibtisam, (2012) and 13%by Omer and Hyafa, (2017). Rajab, (2015) found 13% seroprevalence for CBPP in Western Bahr El Ghazal State, Republic of South Sudan. Recent situation of CBPP in Sudan was estimated in the last project sponsored by African Union (Surveillance of Trade Sensitive Diseases project -STSD project) Table (1.2). More than 8000 serum samples from all states of Sudan were tested at Mycoplasma Department CVRL -2016, Table (1.2).

Table (1.2) Update of CBPP seroprevalence in cattle in Sudan (STSD project)

State	Total samp.	Positive	Negative	positive%
Blue Nile	140	4	136	2.8
North Kurdofan	490	66	424	13.5
West Kurdofan	560	60	495	10.8
South Kurdofan	490	39	451	8
Aljazeera	560	23	537	4.1
Khartoum	420	14	405	3.3
Senar	490	32	458	6.5
AlGadaref	770	91	655	12
North State	350	14	335	4
River Nile	420	16	387	4
White Nile	630	51	576	8.1
Red Sea	490	20	466	4.1
Kassala	770	47	653	6.7
North D.	350	91	259	26
East D.	280	5	275	1.7
West D.	351	25	326	7.1
South D.	350	35	315	10
Central D.	280	75	205	26.7
Total	8121	708	7358	**8.7%**

Source:CVRL-Sudan (2016)

1.2. Aetiology

Contagious Bovine Pleuropneurmonia was the first mycoplasmal disease recognised (Nocard *et al.*, 1898), it was named *Asterococcus mycoides* in the early twentieth century (Provost *et al.*, 1987). Then it was definitively classified as a *Mycoplasma* and renamed *Mycoplasma mycoides* subsp. *mycoides* (Edward and Freundt, 1956; Manso-Silván *et al*., 2009).

1.2.1. Taxonomy

Mycoplasma is one of several Genera of class Mollicutes which has five Orders: Mycoplasmatales,Acholeplasmatales,Anaeroplasmatales,Haloplasmatales and Entomoplasmatales (Edward and Freundt , 1967).

Mollicutes are parasites or commensals organisms of human, animals, insects and plants. Mycoplasmatales require cholesterol for growth, as well as other genera of Mollicutes, have three layered cytoplasmic membrane,filterable through filter with 450 nm pore diameter and produce typical fried egg appearance by most species.

Classification of Mycoplasma :(Nicholas *et al*., 2008)

Kingdom: Bacteria
Phylum : Tenericutes or Firmicutes
Class :Mollicutes
Order :Mycoplasmatales
Family: Mycoplasmataceae
Genus : *Mycoplasma*
Ureaplasma

Phylogenetically it is a member of the *Mycoplasma mycoides* cluster which are pathogens of ruminants, and include *M. mycoides* subsp. *capri, M.capricolum* subsp. *capripneumoniae, M. capricolum* subsp. *capricolum* and *Mycoplasma leachii. M. mycoides* subsp. *mycoides* large colony has now been reclassified as a serovar of *M. mycoides* subsp. *capri* and *M. leachii* is the new species designation for *Mycoplasma* bovine group 7 (Manso-Silvan *et al*., 2009). These six mycoplasmas share phenotypic and genotypic characteristics that cause cross-

reactions in conventional diagnostic techniques. Members of the "*mycoides* cluster" are pathogens of cattle, sheep and goats. The closest relative to *Mmm* is the *M. mycoides* susbp *capri* (*Mmc*), which is usually found in goats (European Commission 2001). Members of the "*mycoides* cluster" share many biochemicals, immunologicals and genetic characteristics which present difficulties for accurate diagnosis (Cottew *et. al*., 1987).

1.2.2. Morphology

Like other *Mollicutes*, (*Mmm*) organisms are simple, self replicating and wall-less prokaryotic organisms (Razin, 1983). The mycoplasmas are the smallest free-living bacteria. They range from 0.2 – 0.8 micrometer and thus can pass through some filters used to remove bacteria. They have the smallest genome size, as a result, lack many metabolic pathways and require complex media for their isolation. The characteristic feature that distinguishes the mycoplasmas from other bacteria is the lack of a cell wall. Thus, they can assume multiple shapes including round, pear shaped and even filamentous. This organism stains well with Giemsa (Bordet, 1910), Methylene blue (Dienes, 1945) and other Aniline stains (Turner, 1959).

1.3. Epidemiology of CBPP disease

1.3.1 Host susceptibility

Contagious Bovine Pleuropneurmonia is predominantly a disease of the *Bovidae* of the kind Bos (*Bos taurus* and *Bos indicus*) also in water buffaloes (*Bubalus bubalis*) (Santini *et al*., 1992).There is a variation in susceptibility to infection; in general European breeds tend to be more susceptible than indigenous African breeds (Provost, *et al.,* 1987). Animals less than 3 years of age are less resistant to experimental challenge (Masiga and Windsor, 1978). The susceptibility of cattle to infection with (*Mmm*) depends on many factors such as the type of animal husbandry, individual resistance and other factors (Shallali, 1997).

Small ruminants, in particular goats, have also been shown to harbor the causative mycoplasma (Hudson, 1971). Okoh and Ocholi (1986) isolated (*Mmm*)

from an outbreak of disease in sheep in Nigeria. Brando (1995) isolated (*Mmm*) from the milk of sheep with mastitis as well as from goats with pneumonia in Portugal outside the endemic region of CBPP.

Horses, donkeys and pigs are resistant to (*Mmm*) but mice are susceptible by experimental inoculation (Abdulla, *et al*., 1982).

1.3.2 Transmission and spread of CBPP

The disease is transmitted primary by aerosol transmission, it occurs through breathing an infected droplets from infected animal (Tambi *et al*., 2006; Vilei and Frey , 2010).

Factors that enhance the infection rate are closeness of contact, intensity of infection and the level of individual susceptibility (Turner, 1954). Close repeated contact is generally thought to be necessary for transmission; however, (*Mmm*) might be spread over longer distances (up to 200 meters) when the climatic conditions are favorable (Campbell, 2015). Even under extensive conditions the gathering of animals at watering places will lead to outbreaks and will maintain the disease in herds (Coetzer *et al.*, 1994; Ibtisam, 2012).

Chronically infected and symptomless animals play an important role in the persistence and spread of the disease, when chronic carrier animals are subjected to stress, like when mustered or walked for long distances (William and William, 2002; Rovid, 2008). *M. mycoides* can pass through the placenta from the infected dams to the off-spring (Stone and Bygrave, 1969). Another mode of transmission occurs through contaminated fodders with urine (Masiga *et al*., 1972).Young calves (less than one year of age) do not play the role of being reservoirs of infection because they show the infection in the form of an arthritis, rather than respiratory form (Harbi and Salih; 1979, El Mahi; 1980).

1.3.3 Morbidity and mortality:

The disease is highly contagious. Morbidity may be reach up to 100% and the mortality rate with CBPP is quite varied and ranges from 10% to 70%, in various outbreaks (Masiga *et al.*, 1996). However, mortality rates in Africa

typically range between 10-70% in epizootics characterized by low morbidity and low or nonexistent mortality, with the majority of infected animals showing chronic lesions (Masiga et *al;* 1996; Regalla; 1996).The mortality rate may depend on other inter- current factors such as plan of nutrition, level of parasitism, and general body condition (Ameera, 2011).

1.3.4. Risk factors:

CBPP is typical example of multifactorial diseases; where factors such as inter- current infections, crowding, inclement climatic conditions, age, genetic constitution, and stress from transportation are important determinants of the final outcome of infection (Thiaucourt, 2004).

1.4. Incubation period and symptoms of CBPP

The length of the incubation period depends upon the volume of the infective dose, the virulence of the strain, and the immune state of the animal and it can last from a few days up to several months (in occasional instance up to 6 months) (FAO, 2002). Although Turner and Campbell (1937) reported a range of 29 – 58 days and Provost *et al.* (1987) stated 20 to 40 days. In general incubation period ranges between 3-6 weeks and may reach up to 3 months (Thiaucourt *et al.* (2004). In experimental infection, Regalla *et al.* (1994) reported that the disease symptoms appear in cattle 40 days after contact with inoculated animals and these symptoms lasted for 20 days.

Depending on the resistance level of the animal and the intensity of exposure; the disease takes an acute, subacute to chronic, or the acute course is sometimes followed by a chronic stage which may last for two years (lunger) as a latent phase of the disease (FAO, 2003).

The early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy. Animals show dullness, anorexia, irregular rumination with moderate fever and may show signs of respiratory disease, coughing is usually persistent and is slight or dry.

The clinical signs in a typical acute case include a rise in body temperature (up to 40ºC), shallow and rapid respiration in the early stage of the disease, which soon becomes abdominal with painful grunting and dilation of the nostrils. As the lung lesions develop, the signs become more pronounced, with increased frequency of coughing and the animal becomes prostrate or stands with the back arched, elbows abducted and neck is extended- Figure 1.1, (Jean, 2008). There is an excess mucous in the nostrils and frothy saliva around the mouth and occasionally soft cough is heard (Bygrave, *et al.,* 1968; Hudson, 1971).

Sub acute or symptomless form is the most common form (40-50%) and it is less severe form of the acute disease. Lesions are localized in small part of the lung, and it's position cannot be easily located by percussion and auscultation. The only symptom is a rare cough, sometimes new foci of infection are created and acute symptoms set in (Henning, 1956). Animals at this stage are able to transmit the infection (Masiga, 1996).

In chronic cases the only symptom is a cough and loss of body condition. The respiratory signs usually appear when the animal rises or when it suddenly passes from a warm stable to cold weather or when it drinks cold water (Henning, 1956).Sub-clinical and chronic cases of CBPP constitute more than 50% of the animals involved in an epizootic (Bygrave *et al.,* 1968).The chronic form 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 (Thiaucourt *et al.,* 2004).

In calves about one year old or less the clinical picture of the disease in primarily characterized by arthritis (Figure 1.2), and the chest may be free from any lesions (Moulton *et al.*, 1956; Turner and Trethewic, 1961; Simon and Johnson, 1963; Harbi and Salih 1979 and El Mahi, 1980).

1.5 Pathology

1.5.1 Gross pathology

Lesions are mostly confined to the lungs and thoracic cavity and mostly appear in a single lung - left side- (Radostits *et al*., 1994). In a study in Portugal, 95% of lesions were unilateral (Egwu *et al.*, 1996), with the diaphragmatic lobes being more commonly affected than cranial lobes.

Adhesions to the chest causing roughened pleural membranes are common. Many liters of straw-colored pleural fluid can be found in acute cases which makes ideal diagnostic material. The interlobular septa are often distended and lungs show the typical marbled appearance (Figure 1.3), with lung lobules showing great variations in color from red, to grey to yellow, depending on the stage of inflammation associated lymph nodes undergo hypertrophy.

In chronic cases the sequestrum is the main lesion type and consists of necrotic material surrounded by a fibrotic capsule ranging from 10 to 100 mm in diameter (Schnee *et al.*, 2011; Tardy *et al.*, 2011)-Figure 1.4. Necrotic foci have been reported in the kidneys of affected cattle (Hudson, 1971, El Mahi, 1980) and pericarditis is occasionally seen in severe cases (Turner and Trethewie, 1961).

1.5.2. Histopathology

The CBPP lesion comprises in the early stage a bronchiolar necrosis and oedema, progressing rapidly to an exudative serofibrinous bronchiolitis with extension to the alveoli and uptake of alveolar fluid into tissue spaces, lymph vessels and ultimately septal lymphatics (Done *et al.*, 1995).

Bronchitis, bronchiolitis, and alveolitis with predominantly neutrophils and mononuclear cellular response constitute the very early inflammation in *Mycoplasma* pneumonia (FAO, 1997).

CBPP is characterized by substantial unilateral pulmonary necrosis, sometimes sequestration, and marked serosanguinous fluid accumulation in interstitia and pleura (FAO, 1997). Vasculitis appears to be an important component of the pathological changes in this disease, explaining the marked

exudation and pleurisy. Thrombosis can explain ischemic necrosis and infarcts of the lung. Death results from anoxia and presumably from toxaemia (Walker, 1999).

In an immunocytochemical study of CBPP infected Italian cattle, Scanziani *et al.,* (1997) showed that the severity of lung lesions correlated with the severity of changes in the lymph nodes. In the acute stage of the disease specific antigen was detected in the lobular periphery and in the cytoplasm of alveolar macrophages.

In chronic lesions, immune reactivity was seen in the fibrotic areas and in macrophages located in the lobular septa; necrotic debris and macrophages located in the inner part of the sequestra were specifically stained. Immunoreactive material was also seen in the centrofollicular areas of the broncho-associated lymphoid tissue structures and in the lymph node follicles. Furthermore, electron microscopy of the mediastinal lymph nodes of a chronically affected calf showed degenerating Mycoplasmas and a few apparently intact Mycoplasmas in the macrophages. (Done *et al.*, 1995).

Figure1.1. Clinical acute stage of CBPP showing extended neck and limbs abduction *(http://www.fao.org/docrep/004/ac147e/ ac147e00.htm*)

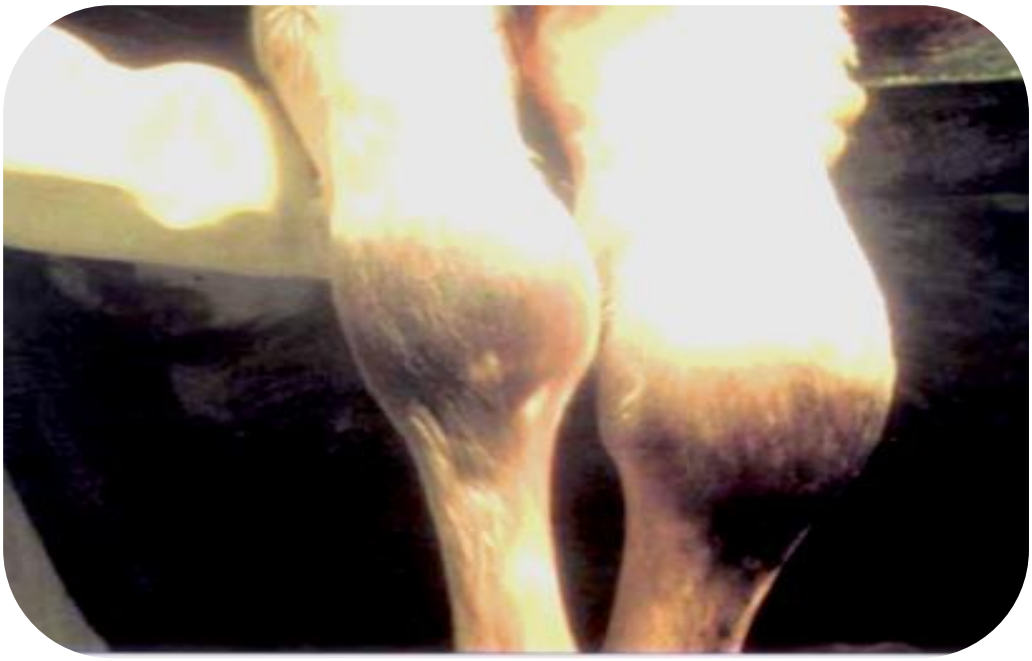

Figure1.2.Swollen knee joint of calf infected with CBPP - acute stage

(http://www.fao.org/docrep/004/ac147e/ ac147e00.htm

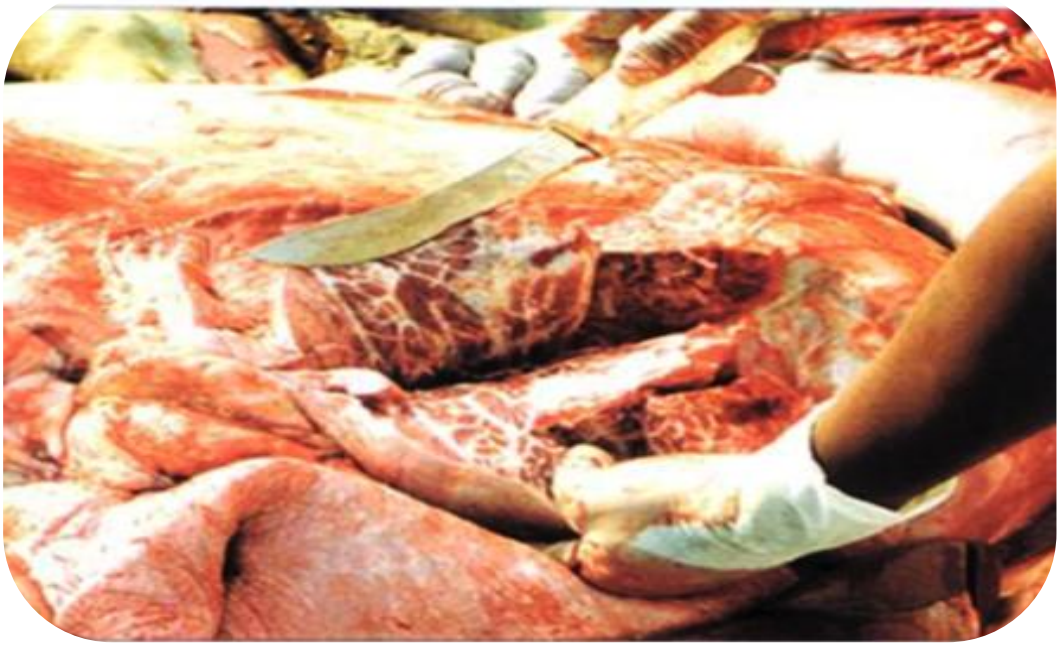

Figure 1.3. Marbled appearance of CBPP infected lung (*http://www.fao.org/docrep/004/ac147e/ ac147e00.htm*)

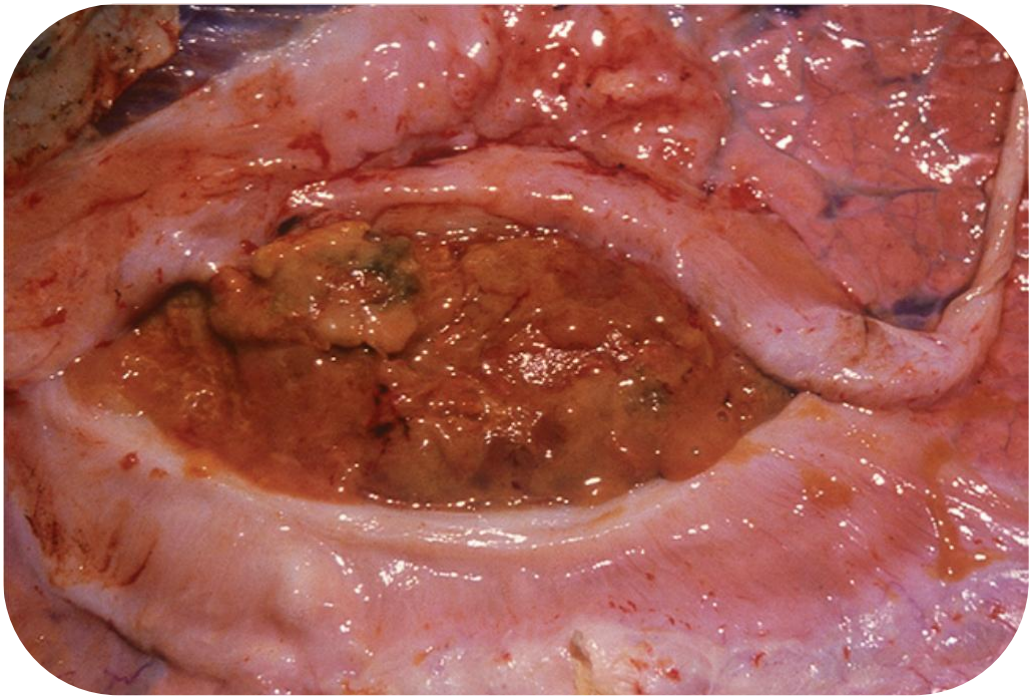

Figure 1.4 Encapsulated sequestra in lung infected with CBPP showed necrotic material surrounded by a fibrotic capsule (*http://www.fao.org/docrep/004/ac147e/ ac147e00.htm*)

1.5.3. Pathogenesis

The disease is typical example of multi-factorial diseases, where factors such as inter-current infections, crowding, inclement climatic conditions, age, genetic constitution, and stress from transportation and handling are important determinants of the final outcome of infection (Rosendal, 1993). The same auther added that an essential part of the pathogenesis of the disease is thrombosis in the pulmonary vessels, probably prior to the development of pneumonic lesions. The mechanism of development of the thrombosis is not well understood, but is considered, at least in part, mediated through induction of cytokines.

1.5.3.1. Capsular polysaccharide:

An important pathogenicity factor in *Mmm* is the capsular polysaccharide (CPS), previously known as galactan (Woubit *et al.*, 2007). 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 *Mmm* have also been associated with the capsule (Nicholas *et al*., 2000).

1.5.3.2. Hydrogen peroxide:

In an investigation reported by Woubit *et al*., (2007), there was indication that glycerol metabolism in *Mmm* strains release hydrogen peroxide (H_2O_2) 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 initial hypothesis was based on the fact that virulent *Mmm* African strains possessed an active ATP-binding cassette (ABC) transport system for the utilization of glycerol, which is metabolized to dihydroxyacetonephosphate (DHAP) releasing H_2O_2, while

European strains lacked part of the glycerol uptake genes due to deletion and are less virulent (Vilei and Abdo, 2000).

1.5.3.3 Variable surface protein:

The *Mmm* express surface proteins which can undergo reversible changes to alter the antigenic repertoire in a cell population (Woubit *et al*., 2007), and this variation enables the Mycoplasma organisms to escape the host immune defense mechanism of their host (Citti *et al*., 2005 , Wise and Foecking , 2006).

1.6. Diagnosis of CBPP:

The diagnosis of CBPP is based on a history of contact with infected animals, immunodiagnosis tests, necropsy findings and cultural examination (OIE, 2008). Table1.3 showed the advantages and disadvantages of some methods used in CBPP diagnosis.

None of the validated serodiagnostic test is sufficient on its own for all the needs of the diagnosis and surveillance of CBPP therefore the use of more than one test is advised (Ronald, 2004).

1.6.1 Clinical diagnosis

It is difficult to distinguish clinically from other causes of respiratory diseases in cattle. Tentative diagnosis depends on clinical signs, post mortem findings and demonstration of *Mmm* in pleural fluid of infected animal by dark field microscopy. Confirmatory diagnosis depends on the isolation of the causative agent and molecular techniques (OIE, 2008).

1.6.2. Isolation of *Mycoplasma mycoides* subsp *mycoides*:

In acute cases the causative agent can be isolated from the blood and nasal swabs. From the dead animal: pleural fluid, portions of affected lungs and lung sequestra (scrapings from inside the capsule) and lung-associated lymph nodes, and kidneys should be taken (OIE, 2008). In chronic cases cultural examination from the sequestrum in Mycoplasma medium usually yields *M. mycoides* (Shallali, 1997).

Table1.3: The advantages and disadvantages of the methods of diagnosis of CBPP

Diagnosis Method	Some advantages	Some disadvantages	Sample types	Preservation
Isolation and identification	diagnosis very sure	Slow and requires viable pathogenic agents	Lungs, pleural fluids	Under cold in order to guarantee the viability of the pathogenic agent
Serology (detection of the antibodies)	Speed and simple	Some tests are not very sensitive and for the majority do not allow to differentiate the post infectious antibodies from the vaccine antibodies	Blood in a tube without anticoagulant (to harvest serum)	Under cold to avoid degradation of the antibodies
Molecular biology	Speed, sensibility and effective even when pathogenic agent is not viable any more in the sample	Expensive and requires a suitably equipped laboratory and adequately trained personnel	Pleural, liquid, lungs, nasal swabs	Does not require any particular precaution
Histopathology	Simplicity and does not require the conservation of cold temperature	Presumptive diagnosis requires some heavy equipment and adequately trained personnel	Tissues (lungs)	In 10% of neutral formal saline

Source: Razin and Tully (1981)

1.6.3. Growth requirements

Because of the Mycoplasmas fastidious nature, samples should be submitted to the laboratory as soon as possible after collection. During transportation, it is advisable to use a transport medium that will protect the organism and antibiotics are used to prevent proliferation of other bacteria such as ampicilin and amoxicillin, which do not affect on the Mycoplasma growth, but affect on other contaminants (OIE, 2018).

The growth medium and nutritional requirements, isolation procedures as well as the physical and biochemical characteristics for the synthesis, culture and characterization of (*Mmm*) have been described by Miles, (1992).

Different Media are available for the isolation and cultivation of *Mmm*. Basically they are composed of two parts; the basic medium and the supplement. The basic part previously used is a broth of lean meat heart muscle or liver (Bennett, 1932; Campbell, 1938; Dafalla, 1961; Gourlay, 1962; El Nasri, 1967 and Buttley, 1967).

The supplement consists of swine or equine serum, yeast extract, DNA, glucose, penicillin and thallium acetate as bacterial and fungal inhibitors.

Nowadays many basic commercial media such as Bacto tryptose, Beef Haert infusion, PPLO and Brucella media are available for isolation and propagation of Mycoplasma. Also the horse serum is brought readymade (Sigma).
After grinding in broth containing antibiotics, the lung samples are diluted tenfold to minimize contaminating bacteria and are inoculated into five or more tubes of broth media and spread it on solid medium. The pleural fluid can be inoculated directly without previous dilution. Growth of this organism in liquid medium culture becomes apparent as homogeneous cloudiness, usually within 2–4 days, frequently with a silky, fragile filament called a‘comet’. During the following days a uniform opacity develops which forms swirls when shaken. On agar media, the colonies are small (1 mm in diameter) and have the classical appearance of ‘fried eggs’ with a dense centre .It is difficult to be seen by naked eye (Edward, 1950).

Different biochemical tests are used to confirm *Mmm* . It is sensitive to digitonin (like all members of the order *Mycoplasmatales*), 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 (OIÈ, 2018).

However, differentiation of *Mmm* strains by serological and biochemical means has been difficult. This difficulty is caused by immunological cross-reactions and biochemical similarities of *Mmm* strains with *M. mycoides* subsp. *mycoides* large-colony (*Mmm*LC). Therefore, immunological test using growth inhibition test and Molecular identification are used as a confirmatory test.

1.6.4. Immunohistochemistry (IHC)

Immunohistochemical examination was used to confirm the presence of *M. mycoides subsp. mycoides* in the lung of domestic buffaloes which had been housed with infected cattle in Italy (Santini *et al.*, 1992) using specific rabbit hyperimmune serum and specific stain. Although the test labours intensive, (IHC) can provide valuable confirmation of CBPP in animals that die suddenly from acute respiratory disease (Scanziani , 1997)**.** On the other hand, the sensitivity of IHC using polyclonal serum can be low and non-specific results frequently occur (Bashiruddin *et al*., 1999). In 2001 report of the EU Scientific Committee on Animal Health and Animal Welfare (European Commission 2001), PCR and IHC were considered tests of choice in presence of carcasses with suspect lesions, when serum was not available and mycoplasma culture from lung tissue was inconclusive because of the poor conditions of the carcass or for logistic difficulties.

1.6.5. Molecular identification - polymerase chain reaction (PCR) test:

PCR is a rapid and sensitive diagnostic method. The PCR- procedure adapted from Bashiruddin *et al.*, (1994). It allows detection of *Mmm* directly in samples of lungs, bronchial lymph nodes, nasal swabs, pleural fluid and blood. It helps in rapid detection, identification and differentiation between members of the

M. mycoides cluster. A number of PCR assays had been developed including real-time PCR which is highly sensitive and specific and less susceptible to contamination than earlier PCRs.

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 (Kasper *et al*., 2005). PCR detection systems based on ribosomal (r) RNA may prove to be more sensitive than those described above because the number of ribosomes is large (about 10^4/cell) resulting in a high copy number of target molecules (Johansson, 1994).

PCR based on the 16S rRNA gene coupled with electrophoretic separation of PCR products on a denaturing gradient gel (DGGE), which can detect most mycoplasmas and even mixed cultures directly from clinical material (McAuliffe *et al.*, 2005). The same author added that this test could differentiate over 70 *Mycoplasma* species (McAuliffe *et al.*, 2005).

1.6.6. DNA sequencing

DNA sequencing today is used chiefly to characterize newly cloned cDNAs; to confirm the identity of a clone or mutation; to check the fidelity of a newly created mutation, ligation junction, or product of a polymerase chain reaction (PCR).

In some cases it used as a screening tool to identify polymorphisms and mutations in genes of particular interest. The sequences are captured automatically and transferred to computers (Joseph S.and David W.,2001).

1.7. Serological diagnosis of CBPP:

Reliable and efficient diagnostic tests for the serological diagnosis of CBPP are the cornerstone of any disease control strategy. The requirements for diagnostic tests depend on the purpose of the diagnostic testing and the epidemiological needs. There are three distinct seroconversion patterns among *Mmm*-infected animals: early high responders, late high responders and low responders. This variability raises questions as to the choice and suitability of current serological tests for single-animal diagnosis. While valid at the herd level, individual test

results 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 (Schubert E. *et al*., 2011)

The following mentioned serological tests are arranged according to the developing time and some of their advantages and limitations.

1.7.1. Tube Agglutination Test:

The tube agglutination test using a formalin-killed suspension of the organism was found unsatisfactory for diagnosis of some of the severe cases and all the carrier animals (Campbell, 1938).

1.7.2. Slide agglutination test:

The slide agglutination test using serum (Priestly, 1951) or blood (Newing and Field, 1953) is sensitive in early stages of the disease and suitable for establishing preliminary diagnosis when large number of cattle is involved and for selection, so it is recommended to be used as a herd test rather than on individual animal. The test has disadvantages of false positive results and false negative in chronic stage of the disease with ease, cheap and rapid advantages. Otherwise the agglutination test is considered to be at least as satisfactory as the complement fixation test for the diagnosis of the disease in chronic cases, as stated by Newing and Field (1953) and may prove more sensitive as Priestley (1951) reported than CFT in reveling evidence of response to vaccination.

1.7.3. Complement fixation test:

The Complement Fixation Test is the approved OIE test. It is a classical serological test designed to measure serum levels of specific antibody to antigens (Campbel and Turner, 1953).

It is thought to be highly specific 98% and sensitive 63.8% (Bellini *et al.*, 1998). In the acute phase of the disease it can detect nearly all sick animals with acute cases (Sacchini *et al*., 2012). It seems rarely detects asymptomatic animals in the early stage of infection or animals with chronic lesions (Nicolas, *et al.*, 1996).

The validity of the results has to be confirmed by post-mortem, bacteriological examination, and serological tests on blood taken at the time of slaughter (Martel *et al*., 2004; Regassa *et al.*, 2005). The test has some limitations, for example it is relatively expensive to perform, slow, and it requires trained personnel and laboratory facilities, and there are a percentage of false negative results.

1.7.4. Precipitation Test:

The precipitation test whether in tubes (Turner, 1962) or agar plates (White, 1958) has proved to be of great value in the examination of suspected sequestra and other lesions (lungs and pleural fluids). This test may be used to detect both antibody in sera and antigen in blood, pleural exudates, body tissue and urine (Gourlay,1965). The same author found this test as accurate as the CFT in detecting clinical signs but less accurate in detecting carriers.

1.7.5. Enzyme Linked Immunosorbent Assays- ELISA:

The enzyme linked immuno sorbant assay (ELISA) was used to detect antibodies against *Mmm* (Onoviran and Taylor Robinson, 1979). The World Organization for Animal Health (OIE, 2008) recommends the use of complement fixation test (CFT) or competitive enzyme-linked immunosorbent assay (c ELISA) as herd-level test. Indirect ELISA has been described for CBPP for nearly 20 years using crude antigens (Onoviran and Taylor-Robinson, 1979; Poumarat *et al*., 1989; Nicholas *et al*., 1996). It appears to be sensitive but may lack specificity.

A competitive ELISA (cELISA) was developed at CIRAD EMVT, Monlpellier (Le Goff and Thiaucourt, 1998), and validation tests have been performed in Africa (Thiacourt *et al*., 1999). This test uses a mouse monoclonal antibody.

The cELISA test has been validated internationally; In May 2004, the cELISA was designated as an OIE prescribed test for international trade by the OIE International Committee (now the World Assembly). The performance of this cELISA method has also been validated by the French Committee for Accreditation in 2009.The test has advantages in term of ease of testing and

standardization of the results no cross reactions with other Mycoplasma species were reported during its development.

It has true specificity at least 99.9% and the sensitivity level is similar to that of CFT (Le Goff and Thiaucourt, 1998). Although Marobela-Raborokgwe *et al*., (2003), stated that the c-ELISA is less sensitive than the CFT.

The c-ELISA was more sensitive in detecting cattle with lesions in the chronic stage than any other test, whilst the CFT detected more during the acute stage (Muuka G. *et al.*, 2011). To achieve maximum sensitivity, the two tests should be applied in parallel (Sidibé *et al*., 2012).

In spite of the fact that cELISA is used for detection of antibodies to *Mmm*, it couldn't, assess vaccination efficiency. This is because post-vaccinal antibodies fail to appear in circulation after 3 months (Le Goff and Thiacourt, 1998).

In natural infection, cELISA can be used for antibody detection even in areas where vaccination against CBPP has been carried out (Provost *et al*., 1987; Le Goff and Thiacourt, 1998; Regalla *et al*., 2000)

An indirect ELISA based on a recombinant protein LppQ-N' had similar sensitivity to the CFT and cELISA. The main advantage; it is stable under harsh conditions (Bruderer *et al.*, 2002).

1.7.6. Latex Agglutination Test

A pen side latex agglutination test has also been developed and is commercially available (Churchward *et al*., 2007). The latex beads were coated with *Mmm* polyclonal immunoglobulin G antiserum and detected *Mmm* antigen in the serum of cattle infected with CBPP, it gives result in less than two minute, using sera or whole blood, and in growth medium containing *Mmm* (Ayling *et al.*, 1999). Confirmation of positive results using other tests is advised.

The development of a LAT that detects this circulating antigen could offer significant advantages in term of diagnosis of early or acute stages of infection (Churchward *et al*., 2007).

1.7.7. Immunoblotting tests (IBT):

It is an immune enzymatic test that described by Goncalves *et al.*, (1998). The IBT is more sensitive and specific test, and is presently used to confirm the results of positive and negative tests in the CFT but requires expert analysis and does not lend itself to mass screening (OIE, 2018). Also Immunoblotting test is used to compare between isolated *Mmm* strains. Gonclaves *et al.* (1998) used the test to detect five different antigens (110, 98, 95, 62/60 and 48 KDa).

Abdo *et al.*, (1998) and Abdo *et al.*, (2000) have identified a 48 KDa protein, named LPPQ. It was found in the type strain of European, African and Australian field strains. They used the protein in an immunoblotting test for the sero-detection of *Mmm* in experimentally infected cattle. To detect latently or chronically infected animals and to differentiate between vaccinated and infected animals almost all serological tests are not suitable but there were some trials to validate skin test (Intradermal Allergic test) for this purpose (FAO, 2003) which relay mainly upon LPPQ(membrane protein) but there is no progress in this trial till now.

1.8. Differential diagnosis of CBPP:

In carrying out a CBPP diagnosis, it is necessary to differentiate this disease from other diseases which may present similar clinical signs or lesions. The following diseases should be considered in differential diagnosis of CBPP (FAO, 1997).

1.8.1. Rinderpest:

The similarity with Rinderpest results from the fever and discharges observed from the eyes, nose and mouth. However, the characteristic lesions of Rinderpest those are essentially erosions in the mouth and throughout the digestive tract, together with the profuse, often bloody diarrhoea 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 and these consist 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.

1.8.2. Foot-and-mouth disease:

Salivation, lameness and fever are the cause of confusion.

1.8.3. Haemorrhagic septicaemia:

It is very acute disease and most affected animals die within 6 to 72 hours after the onset of clinical signs. Buffaloes are particularly susceptible. 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 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.

1.8.4. Bacterial or viral bronchopneumonia:

Clinical signs may resemble closely those of acute CBPP. Post-mortem examination shows usually both lungs to be affected, fibrinous exudates 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.

1.8.5. Theileriosis (East Coast Fever):

Coughing, nasal and ocular discharge and diarrhoea 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 inflammations of the pleura are present.

1.8.6. Ephemeral fever:

In most cases this is a self-limiting disease of short duration; most affected cattle recover quickly, even those which are severely affected. The fever fluctuates with two or more peaks. Pneumonia is not a main feature of the disease but a

secondary pneumonia can occur with lung oedema and emphysema in a small proportion of cases.

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).

1.8.7. Abscesses:

They can be mistaken for sequestra. When cut open the contents of abscesses are seen to be offensive smelling, liquid purulent material, absent in sequestra. In abscesses a total destruction of the lung tissue occurs. Old thickly encapsulated hydatid cysts can also cause some confusion.

1.8.8. 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.

1.8.9. Farcy:

The lung lesions of farcy differ from sequestra as they are filled with foul smelling purulent material (same as abscesses). Similar lymph node lesions are always present.

1.8.10. Actinobacillosis:

The pulmonary lesions, when found, could be mistaken for sequestra. Lesions are generalized and seldom present in lungs.

1.8.11. Echinococcal (hydatid) cysts:

These cysts having a double wall and contain a clear liquid, often calcified when old.

1.8.12. Foreign body reticulopericarditis:

Mostly one animal is affected. The two diseases could be clinically misunderstood, but not epidemiologically and pathologically.

1.9. Contagious Bovine Pleuropneumonia control and eradication

According to the assessment made by the Epidemiological unit of PACE, CBPP is endemic in many parts of Africa. Methods of control depends on: the disease status in a given area, state of the country (clean or enzootic), the mode of animal husbandry (sedentary or nomadic) and the financial status of the country or state or even the owners (Shallali, 1997).

Most African countries including Sudan the most mode of animal husbandry is nomadic, restriction of movement is difficult (Shallali, 1997).

In African countries with endemic CBPP cannot afford eradication by slaughtering of all infected herds (Jores *et al.*, 2008). This was demonstrated by a stamping-out eradication of CBPP in Botswana during 1996, which led to negative effects on short-term economics and increased malnutrition in children who were under the age of five years at the time owing to a total lack of cow's milk. (Boonstra *et al*., 2001).

The disease is very difficult to eradicate and the control measurements depend on routinely serodiagnosis, quarantine of the infected herd, and vaccination (Karib, 1958; Hudson, 1971; Abdulla, 1975). Extensive vaccination programs and chemotherapy are the remaining options for CBPP control in Africa and of these, vaccination still is the preferred method (March, 2004).

1.9.1. Control by vaccination

Louis Willems was the first to attempt the use of a crude vaccine composed of pleural exudate for the control of CBPP in 1850 (Provost *et al*., 1987). After that nomadic Fulanis of Nigeria continue to practice a similar method called "dashe" in which macerated lung tissues from lesions of suspect cases of CBPP are inoculated subcutaneously into the trunk, limbs, nose bridge or tail tip (Herbert, 1974). But the method resulted in serious losses due to post vaccinal reaction in 1991.

The live vaccine preparations produced from one of those strains, KH3J, T1/44 and T1SR (streptomycin-resistant variant) are used in a lyophilized form. The KH3J strain was isolated in the Juba region of Sudan. Since then it has been

passaged 88 times in embryonated eggs, from which broth culture vaccines have been prepared. This vaccine does not cause any adverse reactions in cattle irrespective of route or volume of inocula; however it confers poor immunity lasting only six months. It has been used widely in Western and Central Africa (Rweyemamu *et al.*, 1994).

The OIE recommended vaccine is the naturally mild T1/44 strain which has been passaged in eggs 44 times .It was isolated in Tanzania before more than 40 years ago. It is generally accepted that the protection offered by the vaccine wanes after 12 months (Wesonga and Thiaucourt, 2000) but may last for more than one year (Nkando *et al.*, 2011). It is sufficiently a virulent for *zebu* but can cause severe post vaccinal reactions (Willems reaction) in breeds such as *Bos Taurus* (Teshale, 2005). However some believe that the production of such lesions is essential for protective immunity (Hudson, 1971). The site of inoculation is crucial in order to achieve good immunity while limiting adverse effects, Garba *et al.* (1989) showed that cattle vaccinated with T1\44 by the tail tip produced higher antibody titers and gave better protection than cattle vaccinated by the neck route.

A joint campaign (JP28) against this disease was recommended by Directors of Veterinary Services in African countries at their meeting in Khartoum in 1973 (Abdulla, 1975). It was suggested to vaccinate cattle in East, West and Central African countries using T/1 strain of *M. mycoides* for three consecutive years or more.

Some factors lead to vaccination failure, such as improper storage and handling of vaccines, inadequate dosage and wrong route of inoculation can also be contributory factors in providing poor protection (Shallali, 1997).

1.9.2 Control using chemotherapy

Antibiotic treatment against CBPP is widely used. It is not part of any official control strategy due to suspicion that it could facilitate the development of sequestra, increase the number of carrier animals, increase development of resistant strains, and mask the occurrence of clinical disease (Provost *et al.*, 1987).

However, the method may be of use as it reduces mortalities and bacterial burden (Huebschle *et al*., 2006).

Mycoplasmas are wall-less prokaryotes, so they are resistant to a variety of antibiotics including penicillin and related analogues. Ayling *et al*., (2000) carried out an *in vitro* trial to study the effects of five commonly used antibiotics on a number of strains of *Mmm*, and concluded that tilmicosin and danofloxacin were effective both in terms of mycoplasmastatic and mycoplasmacidal activity; florfenicol and tetracycline provide intermediate effectiveness while spectinomycin was ineffective against some strains. The use of fluoroquinalones, such as danofloxacin, is causing concern amongst regulatory authorities that feel these drugs should be restricted to human use because of rapid increases in microbial resistance. *In vitro* minimum inhibition concentration tests (MIC) indicate tilmicosin, oxytetracycline and fluroquinolones are active against *Mmm* (Ayling *et al.,* 2007). John *et al*., (2012) mentioned that; oxytetracycline, danofloxacin and tulathromycin are all suitable candidates for further investigation as potential treatments for CBPP. Tylosin and spiramycin are effective in the control of excessive vaccination reactions and should be of value in the treatment of clinical cases (Radostits *et al.,* 1994). Animals that does not respond to treatment often become carriers (Nicholas *et al*., 2012).

More work need to be done on the effectiveness of using antibiotics to control CBPP. Undoubtedly treatment of single animal will not control the spread of disease among infected herds, so strategic treatment plans need to be investigated as stated by the FAO-OIE-AU/IBAR-IAEA. Also it is recommended to study the antibiotic resistance in bacteria and any possible subsequent effect on future disease treatment in animals and man.

1.10. Economic importance of Contagious Bovine Pleuropneumonia

CBPP is associated with massive economic losses for cattle keepers (Tambi *et al.,* 2006; Jiuqing *et al.,* 2011). High mortality rate, production loss, increased production cost due to cost of disease control, loss of weight and working ability,

delaying marketing, reduced fertility, trade bans and reduced investment in livestock production all make CBPP economically important. (Radiostits *et al*, 2007).

1.11.Validation of Contagious Bovine Pleuropneumonia diagnostic tests

The validity of a diagnostic test is represented by characteristics, like sensitivity and specificity, and parameters like positive predictive value (PPV) and negative predictive value (NPV) ,(Pfeiffer, 2002).

Sensitivity defines as the proportion of animals with the disease which tested positive, in other words it is the ability to correctly identify diseased animals and therefore gives an indication of how many false negative results can be expected. Specificity is the proportion of animals without the disease which test negative. It represents the ability of the diagnostic tests to correctly identify non diseased animals and gives an indication of how many false positive results can be expected. The increase in sensitivity will often result in a decrease in specificity, and vice versa. Sensitivity and specificity depend on the purpose of the testing. For example, in case of zoonotic or highly contagious diseases a diagnostic test with high sensitivity is warranted. The sensitivity and specificity tests were used to compare the serological tests as done by Matthias *et al*., (2005), who evaluate 12 Commercial serological tests, with PCR which was used as "Gold Standard".

The agreement between serological tests was assessed using the Kappa statistic (Dohoo *et al*., 2003). The kappa test is a statistical method for assessing the agreement between diagnostic methods measured on a dichotomous scale. It measures the proportion of agreement beyond that to be expected by chance. The statistic ranges from 0 to 1, with a kappa value of about 0.4 to 0.6 indicating moderate agreement. Higher kappa values are interpreted as good agreement (Pfeiffer, 2002). Myeong *et al*., (2012) and Schubert *et al.*, (2011) had used this test to compare two Enzyme Immunoassays for detecting *Mycoplasma pneumonia* and four serological tests to detect *Mycoplasma mycoides* subsp. *Mycoides* antibodies respectively. Predictive values are used when taking into account test

characteristics during the diagnostic decision process. They quantify the probability that a test result for a particular animal correctly identifies the condition of interest. The predictive value of positive test stands on the proportion of test positive animals which really have the disease. The predictive value of a negative test is the proportion of test negative animals which really does not have disease. Estimation of predictive values requires knowledge of sensitivity, specificity and the prevalence of the condition in the population. It is important to remember that predictive values are used for interpretation at the individual animal level and cannot be used to compare tests. The predictive values were used by Myeong *et al*., (2012) and Meghan *et al.,* (2018).

If prevalence increases, positive predictive value increases and negative predictive value decreases, and vice versa. Also the more sensitive test, the better is the negative predictive value. The more specific a test, the better is the positive predictive value (Pfeiffer, 2002).

1.12. Calculations for evaluation of the tests and their results

The different parameters which can be calculated for diagnostic tests and their results are summarized in Table 1.4. Rather than memorizing the calculations it is easier to work through them on the basis of their relationship between test results and true disease status using a 2-by-2 table layout as used by Wei Li *et al*., (2015).

Table 1.4. Formulas for comparison and interpretation of diagnostic tests

	Disease	Non Diseased	Total
Disease	a (true positive)	b (false positive)	a+b
Non Diseased	c(false negative)	d (true negative)	c+d
Total	a+c	b+d	N

Sensitivity = $\frac{a}{a+c}$

Specificity = $\frac{d}{b+d}$

Positive predictive value = $\frac{a}{a+b}$,

Negative predictive value = $\frac{d}{c+d}$

True prevalence = $\frac{a+c}{N}$

1.13. Geographical Information System (GIS)

Geographic information system (GIS) is computerized information system that allows for the capture; storage; manipulation; analysis; display and reporting of geographically referenced data. GIS can be used as a tool for any discipline which handles data that can be connected with geographical locations, such as countries, regions, communities, or co-ordinates (Norstrom, 2001). A list of GIS-Application includes: Disease mapping, Epidemiological studies as to show the association between the disease and the hypothesized risk factor and conjunction with software, it can show how disease progress in a population. This method was applied by Gedlu, (2004) and Rajab, (2015); during their serological surveillance.

Maps provide an efficient and unique method of demonstration, distributions of phenomena in the space. Though maps are constricted primarily to show the facts and show spatial distribution with an accuracy which cannot be attained in pages of description or statistics, so their prime importance is as research tools (Norstrom, 2001).The maps were used widely to show the CBPP distribution as in Gedlu, (2004) and Rajab, (2015) studies.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Area of the study and target group of animals

To control CBPP disease it is important to estimate the actual prevalence of the disease and make regular diagnosis programs. Part of the study was directed to show the recent situation of the disease in the Eastern states of Sudan .These states have high population of animals with no vaccination history. which includes: Red sea, Al Gedaref and Kassala States (Figure 2.1) , and to focus on the distribution of the disease within the states and their administration areas (localities), The samples were collected in December-2015 and the samples were collected during the Surveillance of Trade Sensitive Diseases project (STSD), which directed to estimate the actual prevalence of CBPP in IGAD countries. The target study population comprised cattle above six months of age in the selected localities. The status of CBPP in the above mentioned states is unknown since no study had been conducted in these areas. Cattle were selected for sample collection using the simple random method. The herds were either with no vaccination history or had not been vaccinated in the past six months

2.1.1. Locality and animal population of the target study area

Red Sea state Coordinates: 19°35′N 35°37′E. It has an area of 212,800 km² and an estimated cattle population of 142260. Al Gedaref state Coordinates: 14°0′N 35°0′E. It has an area of 75,263 km² and an estimated cattle population of approximately 1088595. Kassala state coordinates: 15°45′N 35°43′E.It has an area of 36,710 km² and an estimated cattle population of approximately 884484 (Ministry of Animal Resources- Information unit , 2015).

2.1.2. Study Design and sample size determination

A Cross-sectional survey was carried out using Geographic Information System (GIS).The sample size required for the study was calculated according to the

formula given by Thrusfield (2005) for simple random sampling:

$$n = \frac{(1.96)^2 \times P\,exp\,(1-P\,exp)}{d2}$$

Where

n= required sample size

P *exp*= Expected prevalence

d = desired absolute precision

Since there has been no research conducted in this area; the sample size was calculated at 95% CI, 5% desired absolute precision and expected prevalence of 50%. Accordingly, the samples collected from Al gedaref state were 770 serum samples, Kassala state were 700 serum samples and 490 serum samples from Red sea state (Tables 2.1; 2.2; 2.3) the total samples were 1960 serum samples.

Figure.2.1. Study area (Red sea, Kassala and Gedarif states)

(Central Veterinary Research Laboratory-Khartoum Sudan. Epidemiology Department)

Table 2.1.Serum samples from cattle in different localities in Al Gedaref state (55 herds)

Locality	Sample size
AlGedaref (wasat) Rural	70
Al Butana	70
Al Gedaref city	70
Al Galabat Eastern	70
Al fashga	70
Al gurisha	70
Basonda	70
Albutana	70
El lfao	70
Al Mafaza	70
El Rahad	70
Total samples	**770**

Table 2.2. Serum samples from cattle in different localities in Red sea state (35 herds)

Locality	Sample size
Dordeb	70
Haiya	70
Sinkat	70
Agig	70
Sawakin	70
Port Sudan	70
Al Gonnob and Awlieb	70
Total samples	**490**

Table 2.3. Serum samples from cattle in different localities in Kassala state (25 herds)

Locality	Sample size
Halfa Elgadieda	70
Naher Atbara	70
Khashm Ghirba	70
Westren Kassala	140
Wad Al Helew	70
Aroma	70
Telkuk	70
Rural Kassala	140
Total samples	**700**

2.2. Isolation and characterization of the *Mycoplasma mycoides* subsp. *mycoides*) (*Mmm*)

2.2.1. Sterilization

All glassware used in this study were soaked overnight in warm 10% detergent solution, brushed and rinsed at least three times in running warm water, followed by rinsing in distilled water. Then were put on clean surface to drain and dry. Graduated and capillary pipettes, petri dishes, forceps, scissors, were sterilized in hot air oven at 160 ºC for 2 hours. Vials such as bijoux, McCartney and universal, were sterilized by autoclaving at 121º C (15Ib/inch) for 15 minute. The plastic bottles used in antigen production were sterilized using autoclave at 121º C (15Ib/inch) for 15 minute.

2.2.2 Types of samples

2.2.2.1. Tissue samples

Pneumonic lung tissue and pleural fluids were collected from animals showing typical clinical signs of CBPP at dairy farm at Soba region -Khartoum state. After postmortem; specimens were cut and placed in sterile plastic bags, labeled and transported directly at 4ºC to the Central Veterinary Research Laboratory (CVRL) - Soba.

2.2.2.2. Serum samples:

Blood samples were collected from the jugular vein, then blood were centrifuged and sera were collected from the blood, sera were labeled and preserved in sterile bottle at - 20˚C.

2.2.3. Media:

Gourlay's media which consist of Bacto tryptose (Oxoid) supplemented with yeast extract (powder), sodium chloride, Disodium hydrogen phosphate, Glucose, Glycerol, horse serum in addition to penicillin and thallium acetate were used, Appendix (1) and Appendix (2).The medium was used as broth or solid media with addition of 1.2-1.5% Agarose.

2.2.4. Cultivation of tissue sample

Lung tissue samples were inoculated in Gourlay's broth media. Approximately **1**gram of the tissues was placed into **9** ml of medium, shaken vigorously and serially 10-fold diluted in to five tubes. Inoculated broth cultures were incubated at 37ºC aerobically for 5 days. The grown broth culture was inoculated on Gourlay's solid media and incubated at 37ºC aerobically in a humid chamber for 5 days. Direct culture was done on the solid medium surface using small piece of lung tissue. The growth in broth media was indicated by the turbidity of the media within 7 days, and change of the color to orange; due to glucose fermentation (compared with the control media). The surface of solid media was investigated for fried-egg appearance of colony under low power lens of stereomicroscope.

2.2.5. Culturing in media without antibiotics:

Following the initial isolation in media containing penicillin and thallium acetate, the samples were cultured onto Gourlay's solid media free of antibiotics, to exclude L-form bacteria. Blood agar also used for this purpose.

2.2.6. Purification and storage of isolate:

Cloning and purification were performed by selection of one pure colony and transferred it several times in to fresh broth and agar media (Tully, 1983[a]).

The mycoplasma isolates can easily lose their viability, so the isolate was lyophilized after addition of skimmed milk using LYOTRAP lyophilize machine, and stored at -20°C.

2.2.7. Identification of the isolates:

2.2.7.1. Microscopy:

Broth cultures were investigated by dark field microscopy. A drop of culture was put on clean slide and covered with a cover slip. The slide was then examined under oil emersion lens. Short or long filaments indicated the presence of Mycoplasma.

2.2.7.2. Cultural characteristics:

Growth in liquid and solid media was fully described in (2.2.4).

2.2.7.3. Biochemical tests:

Biochemical tests performed were: Glucose Fermentation, Arginine Hydrolysis and Reduction of Tetrazolium. Appendix (3).

The Digitonin test was performed by using the drop technique. The sensitivity to digitonin indicates the requirement for sterol; members of the genus Mycoplasma are digitonin sensitive. The digitonin induces lysis of mycoplasma cells via formation of digitonide-cholesterol precipitation complexes which lead to increase the permeability of mycoplasma cells membrane. A drop of culture was placed on the surface of medium and allowed to run in one way. Then a disk of digitonin was placed aseptically on the line of culture. After aerobic incubation at 37 º C in humid chamber for 7 days, disappearance of growth only around the disk indicated the sensitivity of the isolate to digitonin. Appendix (3).

2.2.7.4. Growth inhibition test (immunological tests):

Growth inhibition test (GIT) was used for isolates identification as described by Wallace and Clyde (1983). The GIT stems from the fact that high titers antiserum added in to mycoplasma growth medium inhibit the growth of the mycoplasma species against which the antiserum was produced.

About 0.1ml of the broth culture was deposited on the Mycoplasma agar media and allowed to run across the plate. The plate was then left at room temperature until the entire inoculums was absorbed into the medium. The sterile disk (containing *Mmm* antiserum) was placed carefully on the running drop line using alcohol flamed forceps. The plate was incubated aerobically in a humid champers at 37 ºC for 4 days. The surface of the plate was then examined using stereomicroscope on low magnification for evidence of a zone without colonies encircling of the disk. Also a well was made in the middle of the running drop culture and filled with 20µl of antiserum and refilled with the antiserum next day and re incubated at the same conditions for more visible results.

2.2.7.5. Molecular characterization:

2.2.7.5.1. DNA extraction:

DNA was extracted from the mycoplasma culture using the Guanidine chloroform method as described by Ciulla *et al.*, (1988) and Alsadig *et al.*, (2014) with minor modification as fellow; the mycoplasma cell cultures were washed with 4 ml phosphate buffer saline till become clear and to insure removal of all the broth media of the culture. Washing was done using centrifugation for 15 minutes at 3000rpm.

Samples in 15ml falcon tubes were subjected to digestion by adding 1ml White blood cells lysis buffer, 10µl of 20mg/µl proteinase K, 300 µl of 7.5M Ammonium Acetate, and 1ml of 6M guanidine chloride then samples were incubated at 37˚C overnight. On the following day samples were cooled down on the bench to room temperature and transferred into another 15ml falcon tubes containing 1ml pre-chilled Chloroform and centrifuged at 3000 rpm for 20 minutes. Three layers were separated. The supernatant was collected to a new labeled falcon tube .10 ml of pre-chilled absolute ethanol was added and mixed gently by moving the falcon tube back and forth quickly. Samples were put into a -20˚c freezer for 24 hours. Then, samples were subjected to quick vortex for one minute. Samples were centrifuged for 15 minutes at 3000 rpm and the supernatant were discarded. Washing twice with 4ml 70% ethanol was performed and after each wash the supernatant was drained with much care to avoid losing of the DNA pellet at the bottom of the falcon tube. The falcon tube was inverted upside down on a tissue paper leaving the pellet to dry from alcohol for at least two hours. Finally, the DNA pellet was re-suspended in 200µl of deionized water and was put into 4˚C for one day to insure total dissolving of the pellet. Vortexing was applied gently and the DNA was transferred into a new 1.5 Eppendorf tube.

2.2.7.5.2 - PCR reaction using *M. mycoides* cluster primer pairs:

A pair of primers was used according to Dedieu *et al*., (1994) method. Their sequences are:

MYC1-F: 5'-TTCTAAATTAGTTACTCGTGCA-3'

MYC2-R: 5'-AATAAACTGTATTCTCTAGCCA-3'.

2.2.7.5.2.1. Master Mix

Master mix was prepared containing all components except, $MgCl_2$ and the DNA sample. The reaction mix final volume was 50 µl, containing 31.25 µl DNase free distilled H_2O, 5 µl ViBuffer A (10X, Vivantis), 0.5 µl dNTP mix (100µM, Vivantis), 0.3 µl of each primer (Sigma), 0.4 µl Taq polymerase (2 units, Vivantis), 2.25 µl $MgCl_2$ (2.25 mM, Vivantis) ,10 µl of the DNA sample (5 µg).

2.2.7.5.2.2. Amplification conditions:

The PCR was performed with a (Techne-TC-512) thermocycler. The heat lid temperature was adjusted to 104°C. The thermocycling conditions consist of 30cycles.The steps were; initial denaturation step of 2 min at 94° C;, annealing at 55°C; final extension step of 7 min at 72°C and hold at 4°C (Dedieu, *et al*., 1994).

2.2.7.5.3. PCR reaction using *M. mycoides* specific primer pairs:

Amplification of the MSC gene of the mycoplasma genomic was done using primers MSC-F: 5'ATACTTCTGTTCTAGTAATATG3'

MSC-R: 5'CTGATTATGATGACAGTGGTCA3'.

PCR amplify band size of 275 bp. PCR was also performed using the one step (single tube) in a 25µl final volume using iNtRON's Maxime PCR PreMix Kit (iNtRON i-Taq, South Korea).

2.2.7.5.3.1. Master Mix:

As the former components but the primer pairs were changed.

2.2.7.5.3.2. Amplification conditions:

Samples were applied for initial denaturation for 5 minutes at 95°C, followed by 14 cycles of denaturation at 94°C for 30 seconds, annealing at

63.5°C for 30 seconds, decrease for 0.5 per each cycle and elongation at 72°C for 1 minute. Then after the completion of the 14 cycles another denaturation at 95°C for 30 seconds and annealing step at 56°C for 30 seconds followed by elongation step of 72°C for 30 seconds, steps were repeated for 19 cycles then a final elongation step was done at 72°C for 15 minutes. PCR was performed on a thermocycler (Senso Quest brand, Germany). Distilled water was used as negative control for each primer.

2.2.7.5.4. Visualization of PCR products:

After PCR amplification of each gene, the PCR products were separated on a 2% agarose gel (iNtRON biotechnology, South Korea) in 1X TBE buffer and stained with 3µl Ethidium Bromide. Five µl of each product was added to 2µl of bromophenol dye and then loaded on the gel and left to run in gel electrophoresis system (Bio-RAD Brand, USA) for 90 minutes at 100V. The PCR product was visualized by ultraviolet trans-illuminator (Bio.Doc-it UVP, Cambridge, UK). The molecular weight of DNA bands was estimated in relation to standard 100bp DNA ladder.

2.2.7.6. Sequencing of 16sRNA gene

DNA purification and standard sequencing was performed by Macrogen Company (Seoul, Korea).

2.2.7.7. Bioinformatics analysis

The chromatogram was viewed by Finch TV program, (http://www.geospiza.com/Products/finchtv.shtml). Then the nucleotides sequences were searched for sequences similarity using nucleotide BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi.) (Atschul *et al.*,1997). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using BioEdit software (Hall, 1999).

2.3. Serological tests:

2.3.1. Latex Agglutination Test (LAT)

2.3.1.1. The reagents: are illustrated in Appendix (4)

2.3.1.2 .Method

A White LAT kit for the rapid detection of antibodies against *Mmm* (BoviLAT RAI6223-.Animal Plant Health Agency-Weybridge) was used. The kit was used after reach to the ambient temperature. Then 20µl of the antigen was mixed to the same volume of tested sera, they were mixed together with pipette tip. The agglutination was recorded after 2-3 minutes.

2.3.2. Competitive ELISA test

2.3.2.1. Biological and chemical reagent for c-ELISA: are illustrated in Appendix (5).

2.3.2.2. Principles and method

Serum samples were diluted and incubated with the specific monoclonal antibody (117/5) in the pre-plate. The mixture was transferred into the *Mmm* coated micro plate. Any antibody specific to *Mmm* in the serum will form an *Mmm* bovine antibody immune complex, which effectively masks the *Mmm* sites. In this case the monoclonal antibody cannot bind to the corresponding epitope. After washing, an anti-mouse-IgG antibody coupled to peroxidase was incubated in the wells. In the presence of specific *Mmm* antibodies in the serum that is being analyzed, the monoclonal antibody (117/5) is not fixed in the plate and the conjugate cannot bind in the wells. On the contrary, the conjugate can bind to the monoclonal antibody. After washing, the enzyme substrate (TMB tetramethyl benzidine) was added to the conjugate, forming a blue compound becoming yellow after blocking with stop solution. The plates were read by the c-ELISA reader at 450 nm mab and negative controls exhibit a dark yellow color while strong positive serum controls are very pale yellow color. The cut-off point has been set at 50%.The cut off point was calculated using a monoclonal control (CMab) as the formula: PI E%=100× (CMabx-EA(450))/(CMabx-CCx).

2.4. Development of the Stained Slide Agglutination Test from reference and RH local isolates

The steps are illustrated in Table 2.4.

2.4.1. Working seeds production

The T1\44 reference strain was obtained from PANVAC reference laboratory (Ethiopia) as vaccine strain. The antigen was produced from the working seed in broth medium.

Local strain (RH) was isolated and confirmed previously by conventional, serological techniques and by polymerase chain reaction. Sterility, purity, identity and viable count, had been done as a quality control tests (FAO, 1997). The isolate was lyophilized and stored at -20 º C as a working seed.

2.4.1.1. Sterility test:

Sterility test was done by inoculating 0.5 ml of the culture in 2 tubes of thioglycollate, incubated at 37 ^{o}C to detect aerobic and anaerobic bacteria, and 3 tubes of soy bean digest medium. The two tubes were incubated at 37 ^{o}C and one tube was incubated at room temperature for 2 weeks to check for the presence of aerobic bacteria and fungi. The Blood agar was used for the same purpose.

2.4.1.2. Identity test

It was performed by using specific antiserum (GIT) as mentioned in 2.2.7.4.

2.4.1.3. Viable count of the culture:

The viable count of the culture was done by 10 fold serial dilution of 12 tubes with Gourlay's broth, which led to 10^{8} CCU (color change unit).

2.4.2. Preparation of mycoplasma-stained color antigen

The preparation was guided by Newing and Field (1953), Morgan *et al*., (1978) and Arefin *et al*., (2011) The reference and local working seeds were seeded at 1:10 in Gourlay´s broth medium and incubated at 37°C for 8-10 days to reach to the optimum growth. Then Rose bengal stain (1%) was added (1 ml of diluted stain to every 100 ml of culture) and Phenol saline (0.5%) was added

(5ml/L) for inactivation. The flasks were agitated and incubated for 24 hours at 4ºC. After that they were centrifuged at 4,000 rpm for 60 minutes. The supernatant fluid was decanted and the cells washed twice; using PBS (pH 7.2) - (Appendix 6).

2.4. 3. Titration of the antigen

The concentrated cells (packed cells) were diluted to reach to the best visual result using PBS (pH 7.2). These dilutions:- 90%, 70%, %50, %30% and10%, were used.

2.4. 4. Slide micro-agglutination test procedure

For using slide micro-agglutination antigen, 20 µl of stained antigen and 20 µl bovine sera were placed on an enamel plate by microtitre pipette and mixed thoroughly by gentle shaking. The results were read within 2-3 minutes. The antigen produces distinct tiny pink clumps with positive serum.

2.3. 5. Stained slide agglutination test reaction

The reaction of the stained antigen was graded as the Latex agglutination antigen (March *et al*., 2002).

Table 2.4: Description of the Slide Agglutination Test steps

Steps	Method	Technical notes
1-Strain	➢ The T1\44 reference strain ➢ RH-Local strain The viable count of both was 10^8CCU\ml	-
2-Culture	➢ Seeded at 1:10 in of Gourlay´s broth medium. ➢ Incubated at 37°C for 8-10 days.	Reach the optimal growth; sterility ,identity, viable count
3-Addition of dye	Rose Bengal stain (1%) was added (1 ml of diluted stain to every 100 ml of culture)	-
4-Addition of phenol Saline	Phenol saline (0.5%) was added 5ml/L	-
5-Stirring	Stirring for 24 hours	-
6-Centrifugation	Centrifuged at 4000 rpm for 60 minutes	-
7-Washing	Washed twice, using PBS (pH 7.2)	-
8-Titration of antigen	Dilution with different percentages	The concentrated cells was diluted 30% using PBS
9- Antigen assessment	➢ Using reference positive and Negative controls ➢ compare with Latex Agglutination and c ELISA tests ➢ Test of confirmed serums	

2.4.6. Antigen stability

The developed antigens were checked for capability in sero diagnosis using different temperatures. They were kept at 37ºC and retested daily using reference positive and negative serum (Brought from APHA). At 4ºC the antigens were tested every week using the same reference serums for 3 months, and then monthly checked.

2.4.7. Assessment of antigen

The antigens were tested by adding equal volume of antigen and serum. Reference positive and negative controls were used (brought with BoviLAT kit-Animal and Plant Health Agency- APHA-England).

Also total of 15 serum samples from an infected farm (where RH has been isolated) were tested by SAT_1 and SAT_2 tests .

A total of 222 random serum samples from farms in Khartoum state were tested with the developed antigens.

2.4. 8. Antigen cost

Every one liter is produce (2 ml) of the stained antigen to test (100) samples. The cost was calculated by counting the manufacturing cost.

2.5. Statistical analysis

The following statistical analysis were conducted: specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and kappa agreement tests (Fleiss and Cohen, 1973), to compare the concordance between individual tests which are: SAT_1 (stained antigen prepared from reference strain), SAT_2 (stained antigen prepared from local strain) and Latex Agglutination Test (APHA-England) with the c. ELISA (IDEXX –France) - as a golden test-. Also the developed antigens were compared by LAT test.

CHAPTER THREE

RESULTS

3.1. Seroprevalence of CBPP in Eastern states of Sudan

During this study total of 1960 serum samples were collected and tested using c.ELISA. The distribution of samples collection and result of CBPP seroprevalence within the localities is presented in Table 3.1, Table 3.2 and Table 3.3. The highest seroprevalence of the infection was observed in Al Gedaref state (12%), then Kassala state (6.9%) and lastly, Red sea state (4.1%), Figure 3.1 and Figure 3.2.

As shown in Table 3.1, **10** localities in Al Gedaref state were tested .The highest seroprevalence of CBPP was in Al Galabat Eastern and Al gurisha (17.1%), followed by Basonda (14.3%). The least seroprevalence was observed in El Fao locality (4.3%). The same data is shown in Figure (3.3), which is demonstrated by colors differences .The range of seroprevalence of the disease was mentioned in the map´s key.

In Red sea state, **7** localities were tested (Table 3.2 and Figure 3.4).The highest seroprevalence was shown in Sawakin locality (11.4%), followed by Dordeb (8.6%). No CBPP antibodies had been detected in Agig , Al Gonnob and Awlieb localities.

Table (3.3) and Figure (3.5) show the distribution of the disease in Kassala state, **8** localities were tested. The highest seroprevalence of CBPP was in Naher Atbara locality (17.1%) followed by Wad Al Helew locality (15.7%). No CBPP antibodies had been detected in Telkuk locality.

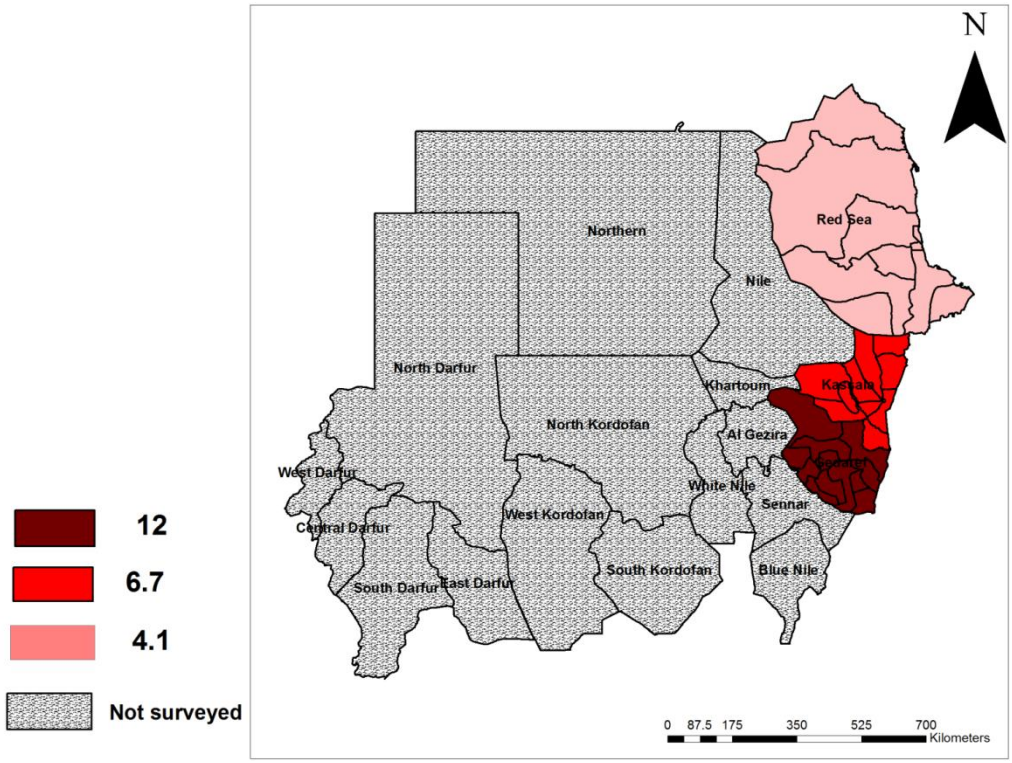

Figure 3.1: Seroprevalence of CBPP (%) in Eastern states of Sudan using c.ELISA test (Central Veterinary Research Laboratory-Khartoum Sudan. Epidemiology Department)

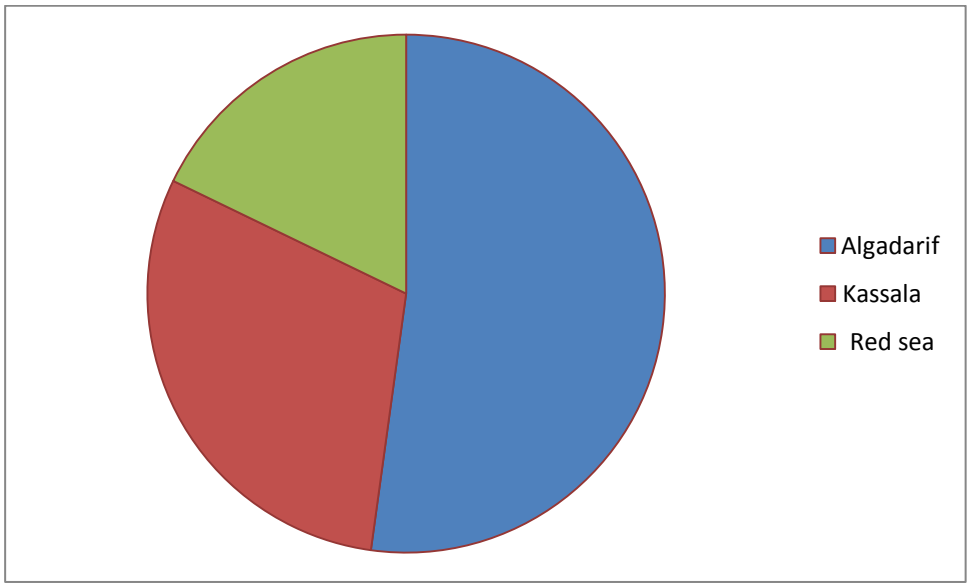

Figure. 3.2: Seroprevalence (%) of CBPP in Eastern states of Sudan

Table 3.1: Seroprevalence of CBPP in Al Gedaref state using c.ELISA test

Locality	Sample size	Positive samples	Seroprevalence%
Al Gedaref wasat (Rural)	70	7	10
Al butana	70	10	14.2
Al Gedaref city	70	8	11.4
Al Galabat Eastern	70	7	10
Al fashga	70	8	11.4
Al gurisha	70	12	17.1
Basonda	70	7	10
Al Galabat Western	70	12	17.1
El Fao	70	9	12.9
Al Mafaza	70	3	4.3
El Rahad	70	9	12.8
Total samples	**770**	**92**	**12**

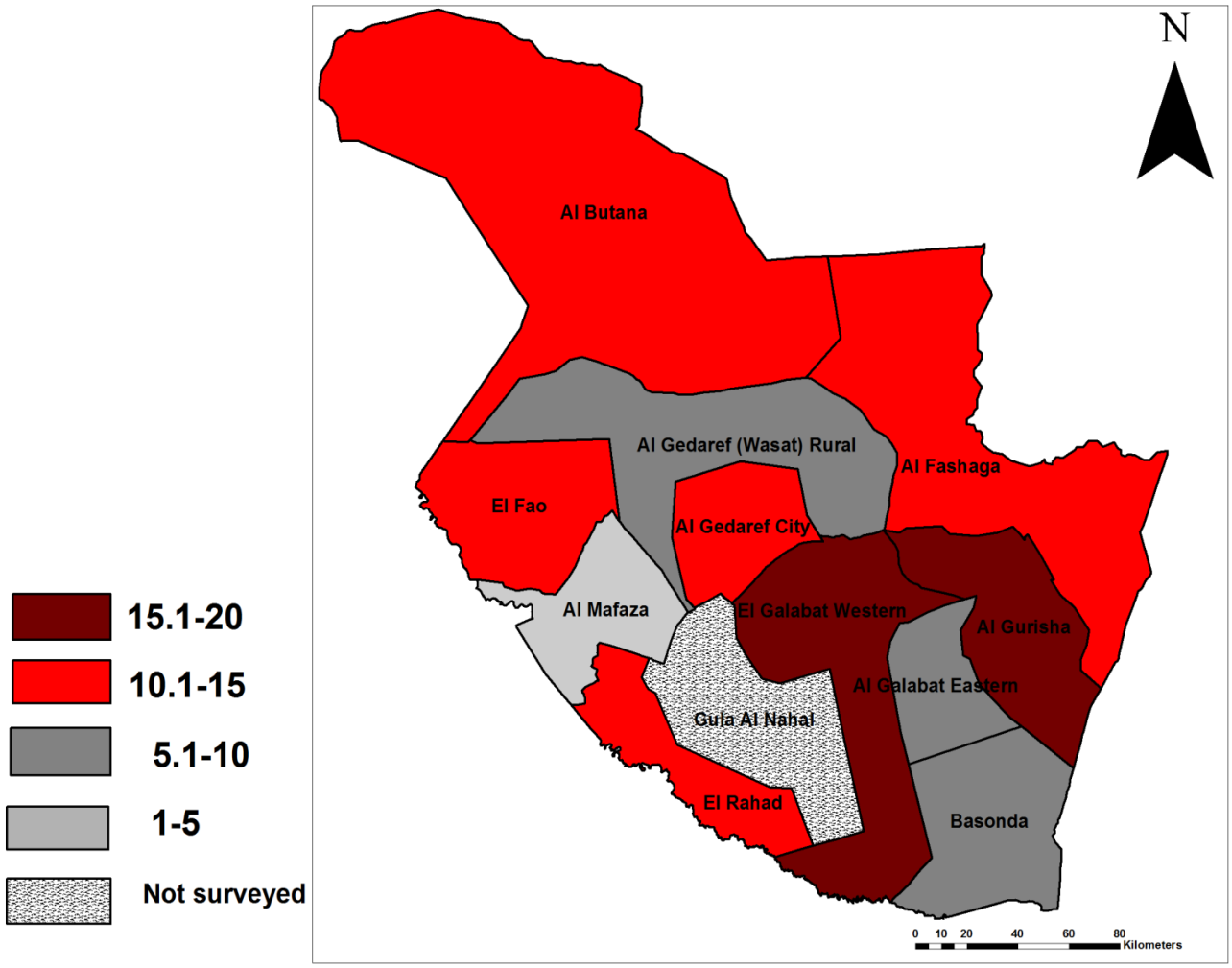

Figure 3.3.Distribution of CBPP (%) within Al Gedaref locality

(Central Veterinary Research Laboratory- Sudan. Epidemiology Department)

Table.3.2: Seroprevalence of CBPP in Red sea state using c.ELISA test

Locality	Sample size	Positive samples	Seroprevalence%
Dordeb	70	6	8.6
Haiya	70	2	2.9
Sinkat	70	2	2.9
Agig	70	-	0
Sawakin	70	8	11.4
Port Sudan	70	2	2.9
Al Gonnob and Awlieb	70	0	0
Total samples	**490**	**20**	**4.1**

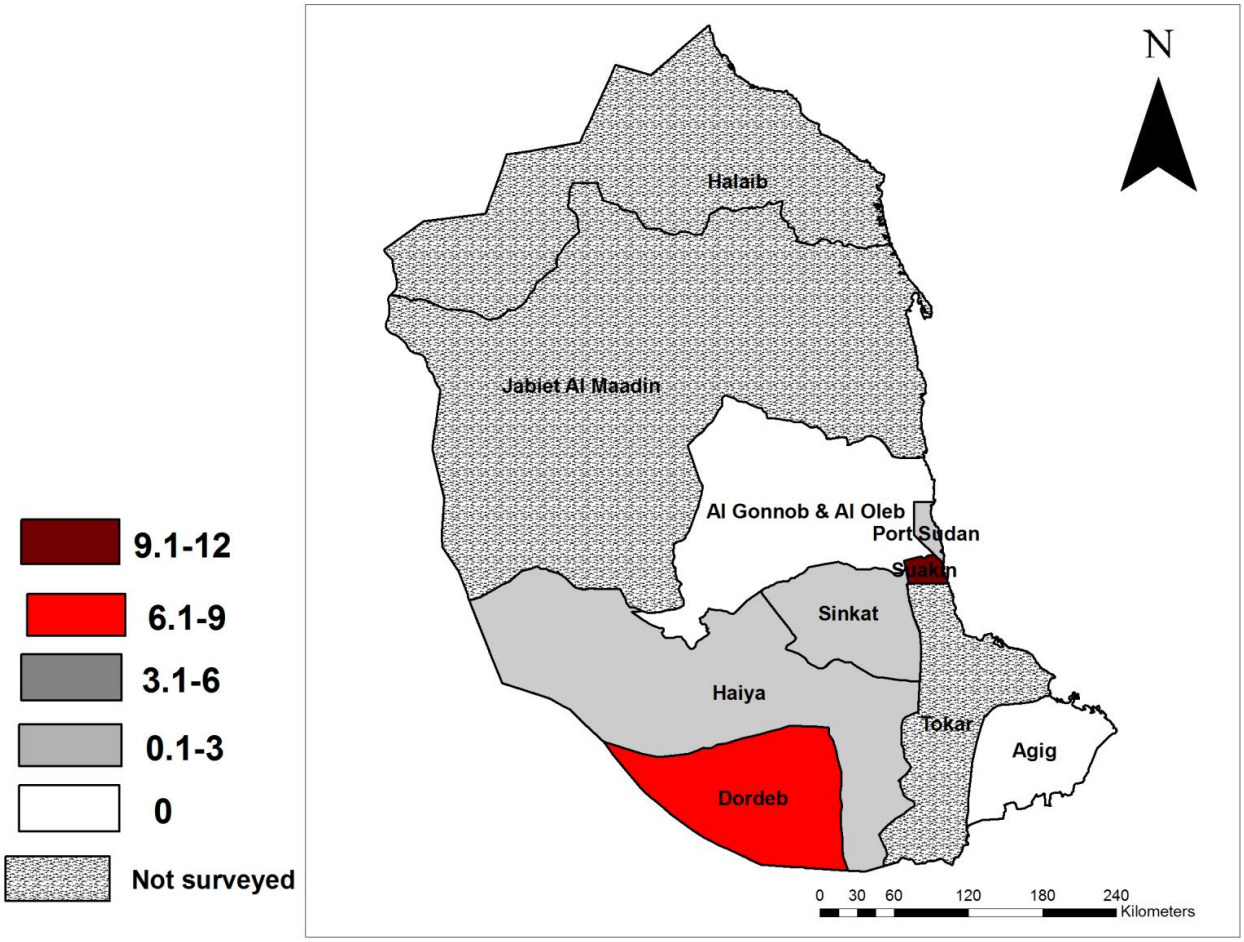

Figure 3.4: Distribution of CBPP (%) within Red sea locality

(Central Veterinary Research Laboratory- Sudan. Epidemiology Department)

Table.3.3: Seroprevalence of CBPP in Kassala state using c.ELISA test

Locality	Sample size	Positive samples	Seroprevalence%
Halfa Elgadieda	70	2	2.9
Naher Atbara	70	12	17.1
Khashm Ghirba	70	7	10
Westren Kassala	140	3	2.1
Wad Al Helew	70	11	15.7
Aroma	70	6	8.9
Telkuk	70	0	0
Rural Kassala	140	6	4.3
Total	**700**	**48**	**6.9**

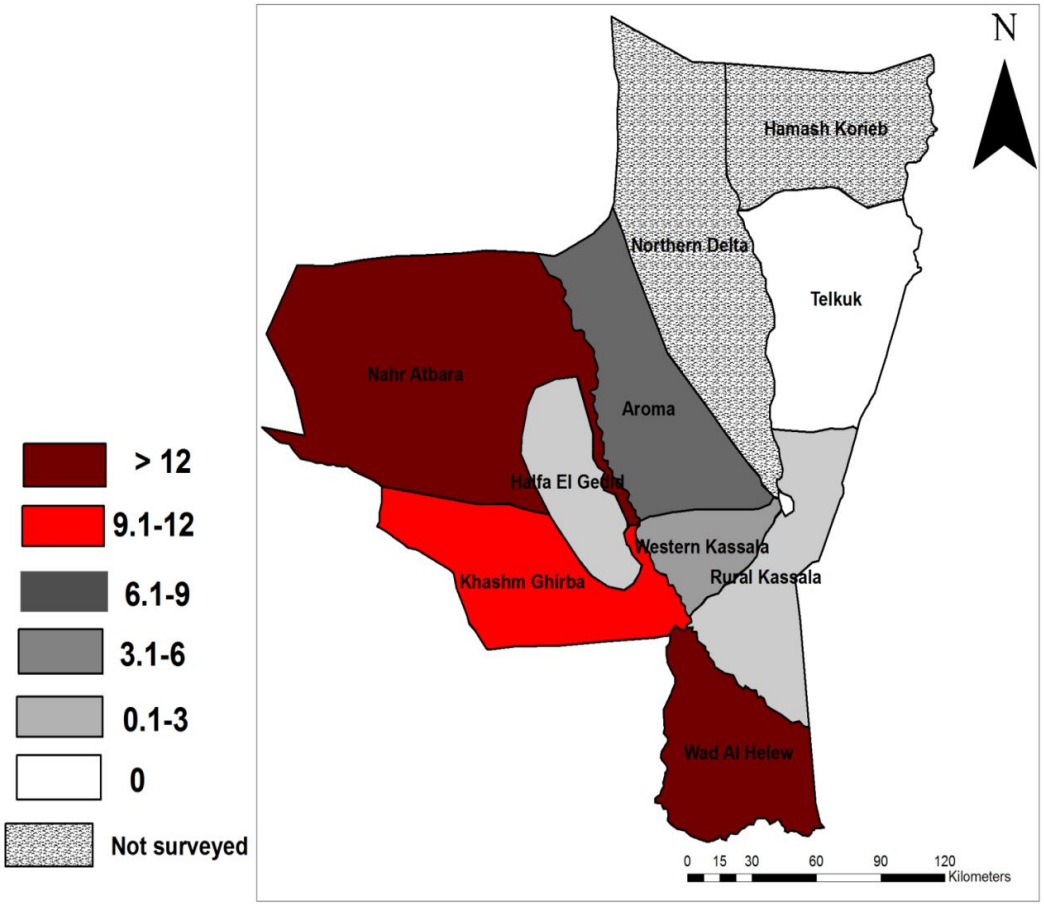

Figure 3.5: Distribution of CBPP (%) within Kassala locality

(Central Veterinary Research Laboratory-Khartoum Sudan. Epidemiology Department)

3.2. Isolation and characterization of *Mycoplasma mycoides* subsp. *mycoides*:

The pneumonic lung samples collected from dairy cattle farm at Soba region showed typical clinical signs like excess mucous secretion from nostrils and difficulties in breathing (Figure 3.6.). Figures (3.7; 3.8) showed on post mortem there were adhesions to the chest, fibrinous tissue on thoracic ribs and hepitization of lung with marbled appearance.

The RH local strain was successfully isolated and identified as *Mycoplasma mycoides* subsp. *mycoides* depending on colony characteristics; fried-egg appearance of colony under low power lens of stereomicroscope was observed (Figure 3.9). In the broth cultures short and long filaments appeared using dark field microscopy. Digitonin test was positive Figure (3.10), there was an inhibitory zone around the digitonin saturated disc; this test confirmed that the isolate is Mycoplasma.

Biochemical tests indicated that the isolate was positive for glucose fermentation test; it changed the color from red to yellow, positive for tetrazolium reduction where the color changed to brick-red and negative for arginine hydrolysis where there was no color change (Figure 3.11).

Growth inhibition test revealed inhibitory zone without colonies encircling of the disk saturated with specific *Mmm* antisera (Figure 3.12), this test is highly specific and confirmed that the strain is *Mmm.*

Molecular test using cluster primer pairs MYC1/MYC2, the RH isolate showed fragment band size on Agarose 574 b.p (Figure 3.13).Using specific primer pair's MSC1/MSC2, the isolate showed fragment band size on agarose gel, 275 b.p (Figure 3.14).

The DNA sequencing for (RH) *M. mycoides subsp. mycoides* strain showed 100% overall identity to two strains the *Mmm* PG1 and *Mmm* Vmm gene strains from gene-bank (Figure 3.15).

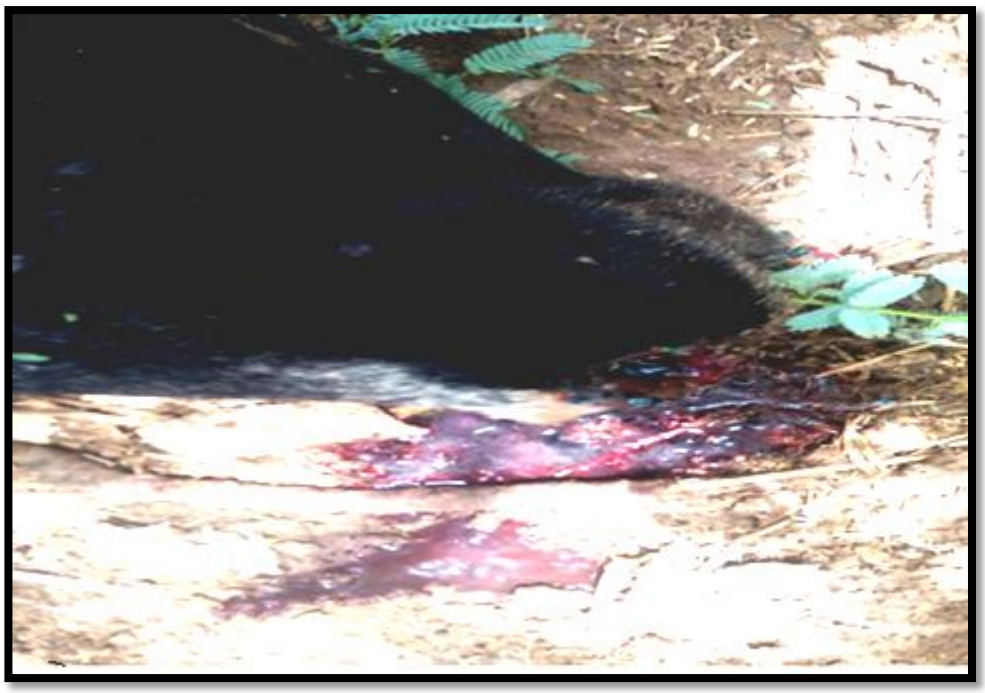

Figure 3.6. Exess mucus secretion from nostrils-Acute stage of CBPP disease

Figure 3.7. Chest lesion of CBPP showing adhesion to the chest and fibrinous tissue on thoracic ribs of CBPP diseased animal

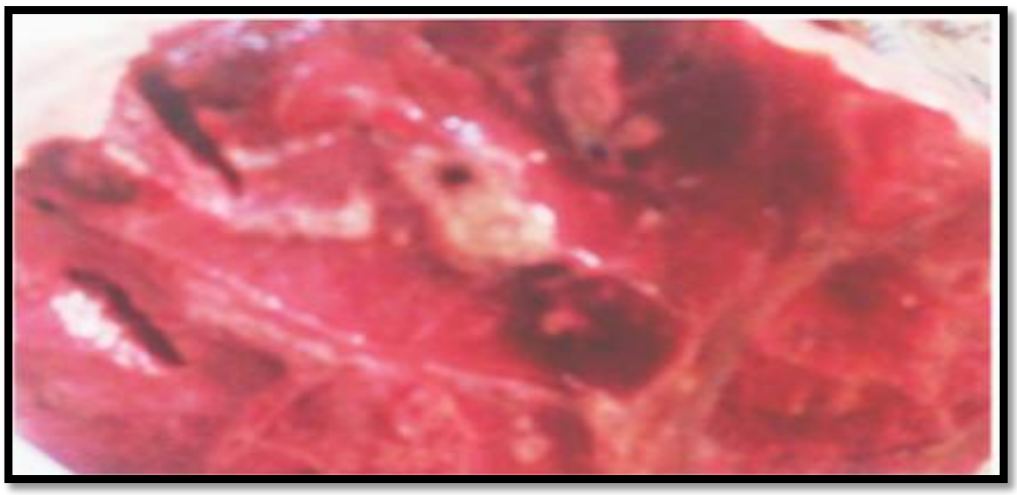

Figure 3.8. Hepitization and marbled appearance of CBPP infected lung

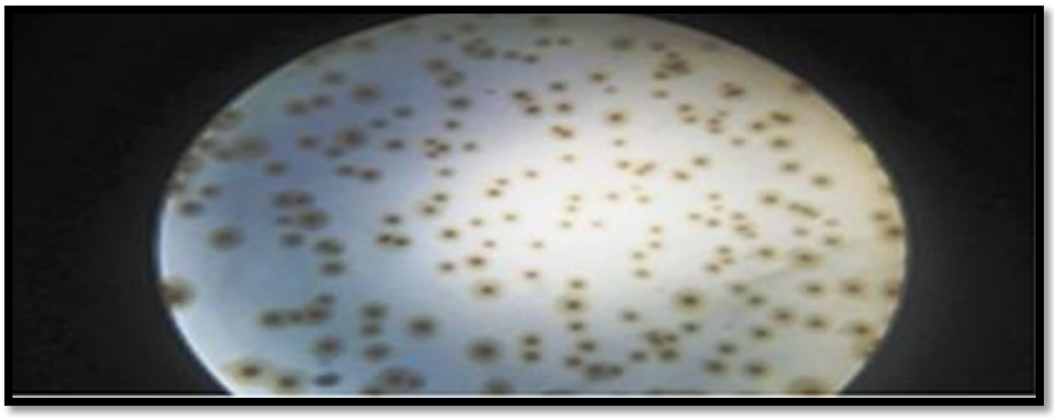

Figure 3.9. *Mycoplasma mycoides* sub sp. *mycoides* colonies showing fried egg appearance

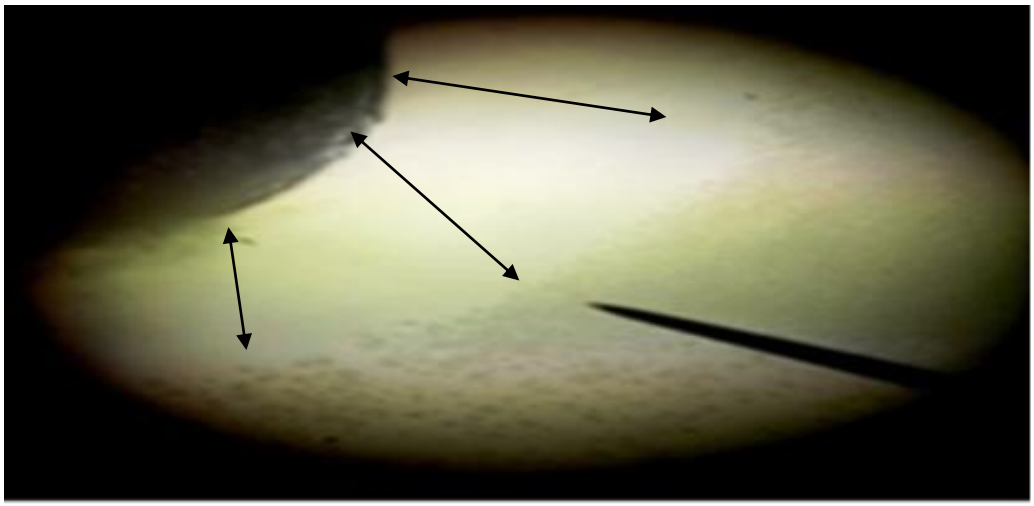

Figure3.10. Digitonin test: The isolate showed inhibitory zone around the digitonin disk

Figure3.11. Biochemical tests for *Mycoplasma mycoides* sub sp *mycoides* isolate

T: Tetrazolium reduction test

A: Araginine hydrolysis test

G: Glucose fermentation test.

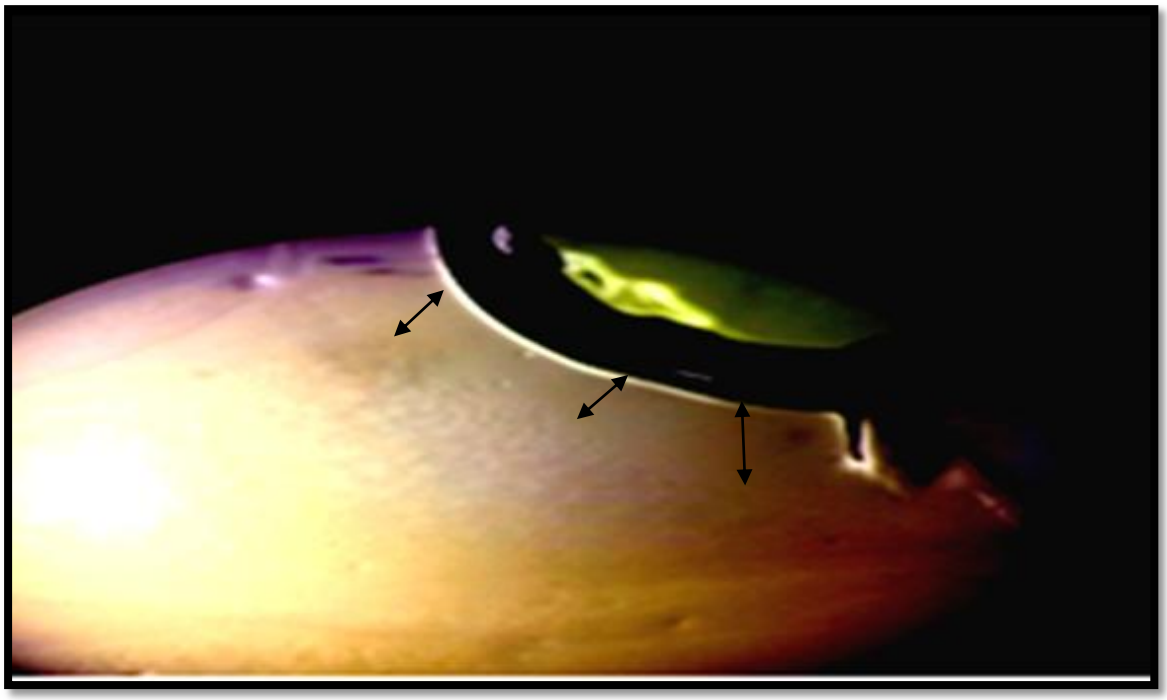

Figure3.12. Growth inhibition test showing inhibitory zone around a well filled with specific antiserum against CBPP

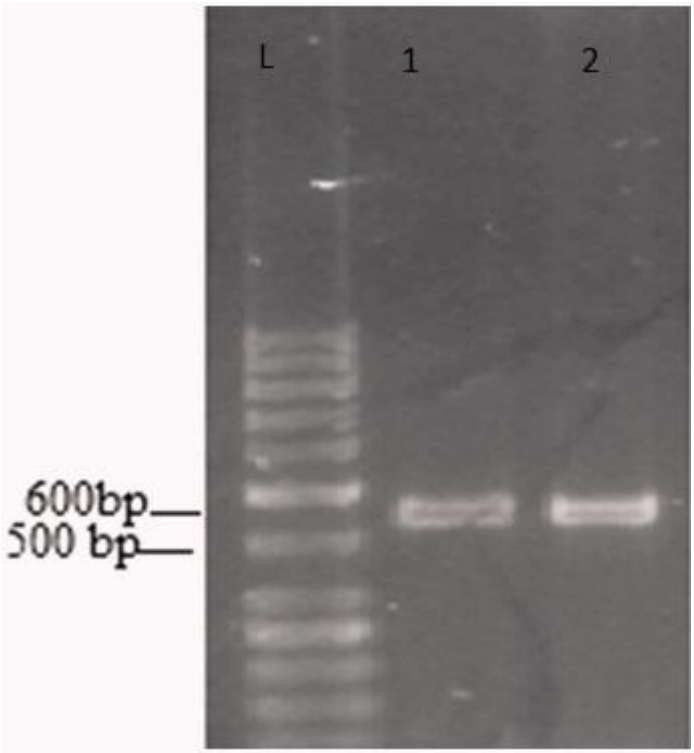

Figure 3.13. PCR reaction using *Mycoplasma mycoides* cluster primer.Lane: DNA ladder 100 bp.Lane1: positive control. Lane2: RH local isolate.

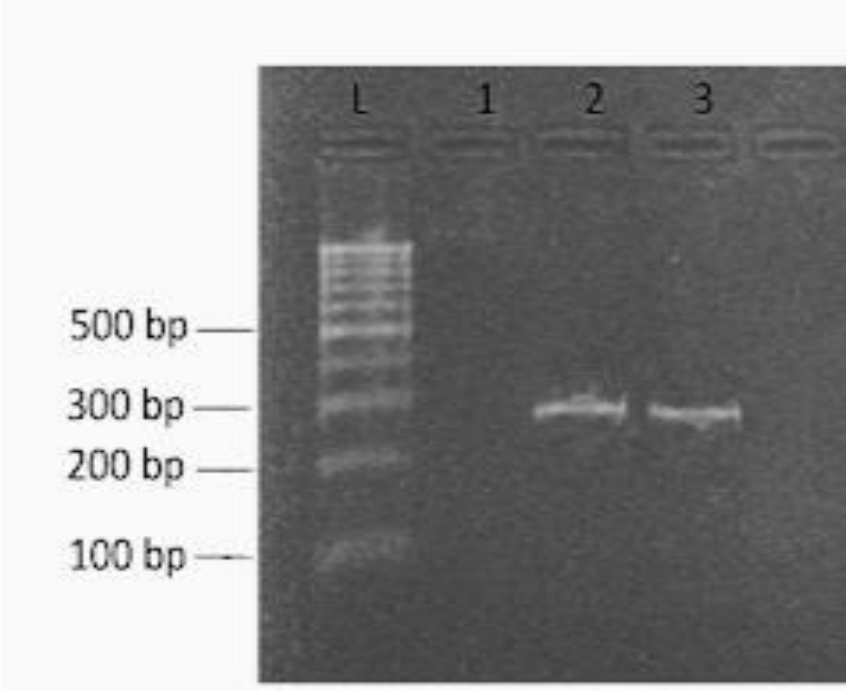

Figure 3.14. PCR reaction using *Mycoplasma mycoides* specific primer.**L**: DNA ladder 100 bp. **Lane1**: Negative control , **Lane2**: positive control, **Lane3**: RH local isolate.

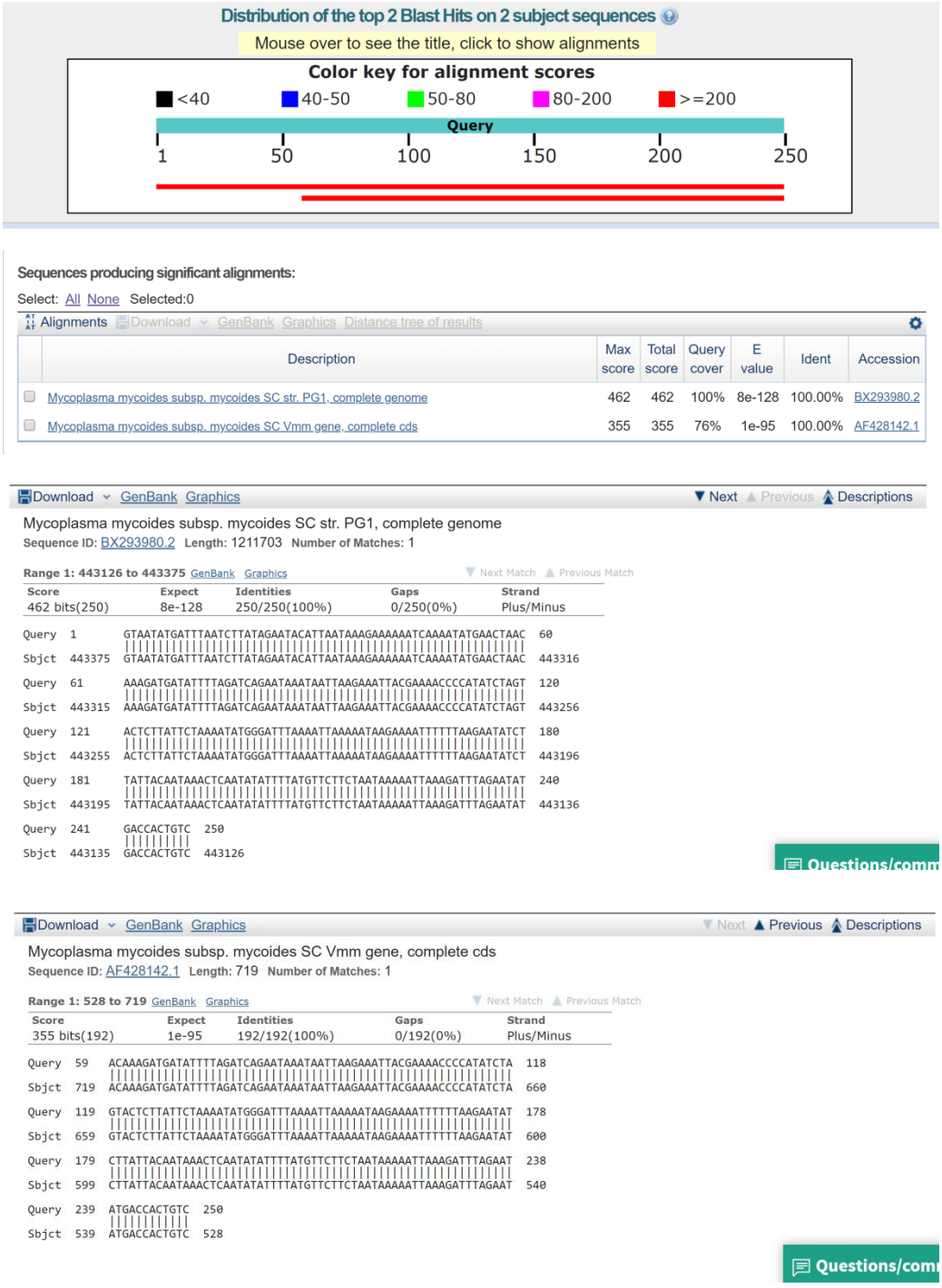

Figure 3. 15: Bioinformatic results showing 100% overall identity to the *Mmm* PG1and *Mmm* Vmm gene strains from gene-bank

3.3. Development of stained antigen

3.3.1. Cultural results

The culture of RH local and T1/44 reference strains was sterile, pure, and identical to *Mmm* .The viable count was10^8CCU/ml. The broth culture formed confluent growth with turbidity and sliminess and the color was changed from red to yellow (Appendix 7).

The Rose bengal-stained antigen (Appendix 7) was produced and the agglutination was clearly seen with reference positive and negative serums.

3.3.2. Stained Slide agglutination test reaction

In positive serums, granules (agglutinates) formed rapidly due to combination of homologous antigen and antibody, which was seen during rocking. The agglutinin was pink with purple background. The reaction is graded as follows: rapid coarse agglutination complete with in 15-30Sec = three-plus, definite reaction but far from complete= two plus, very fine agglutination recognizable in a good light= one plus. No agglutination was found in negative samples (Fig.3.16). In comparison; Figure (3.17) showed the agglutination grades of Latex agglutination test antigen (APHA-England).

3.3.3. Antigen stability

The antigens were kept at 37ºC and found to be stable for 48 hours; they gave the same result with the standard serum (positive and negative). The antigens were also stable for one year when kept at 4 ºC and tested with the same standard serums (positive and negative).

3.3.4. Titration of the antigen

Every 1 liter of broth culture was found to produce 2ml from stained antigen. The packed cells were diluted using PBS as diluent (90% 70% 50% 30% and 10%). It was found that 30% concentrations give the most clearly visible agglutination. While in the other concentrations the agglutination was not clear (Fig.3.18), more than 30% was dense and less than 30% was light and difficult to see the agglutination.

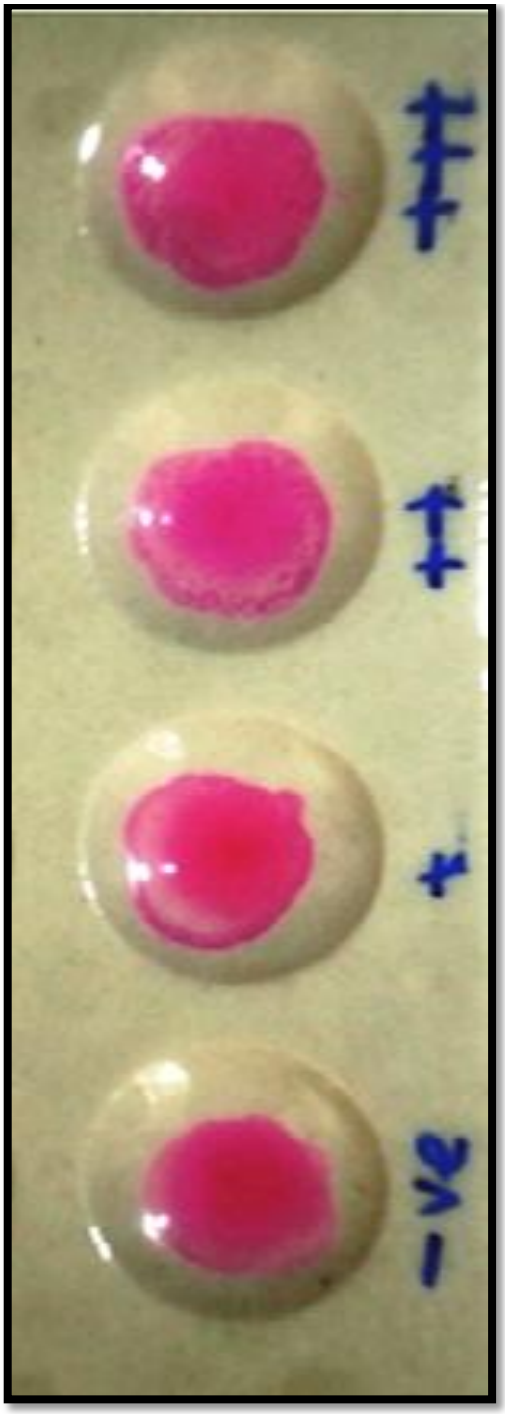

Figure 3.16. Agglutination grades of the CBPP stained antigen:

+++ :Rapid coarse agglutination
++ :Definite reaction but far from complete
+ : Very fine agglutination
-ve: No agglutination seen

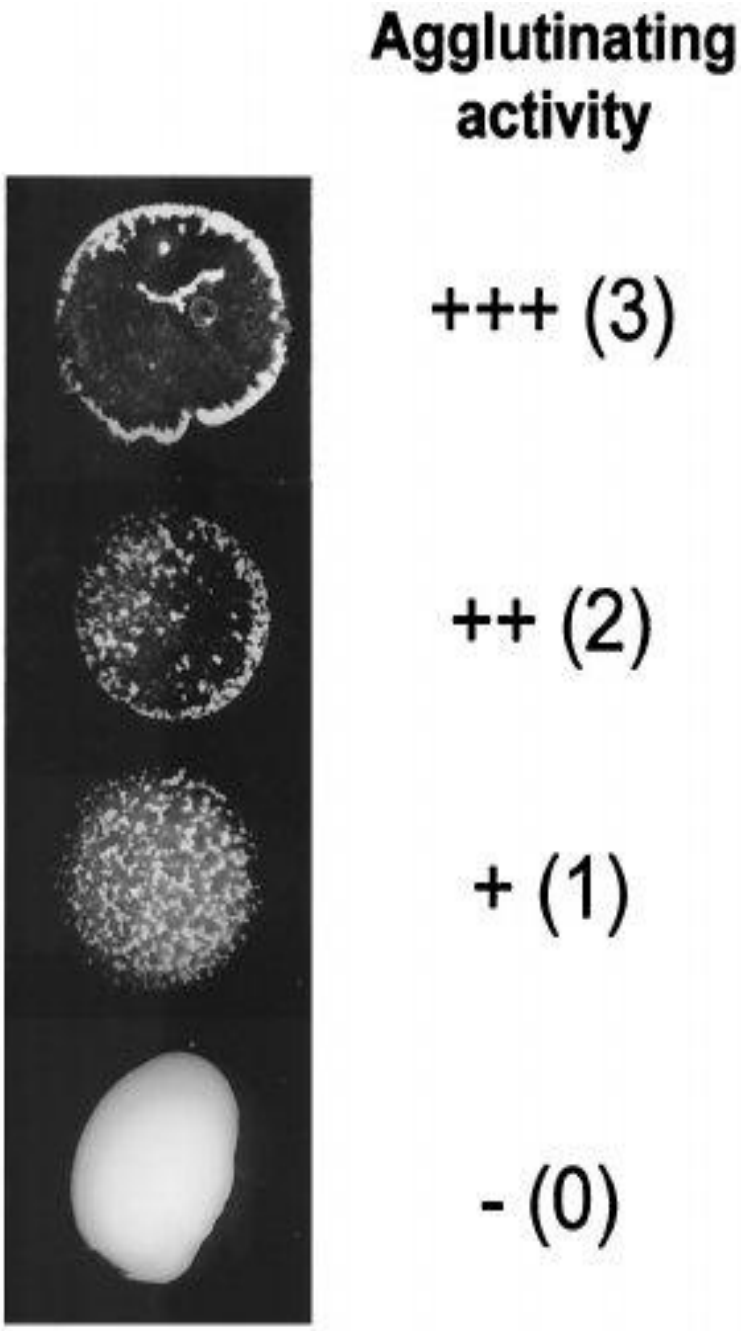

Figure 3.17. Agglutination grades of Latex agglutination test antigen for CBPP serodianosis (www.citeseerx.ist.psu.edu).

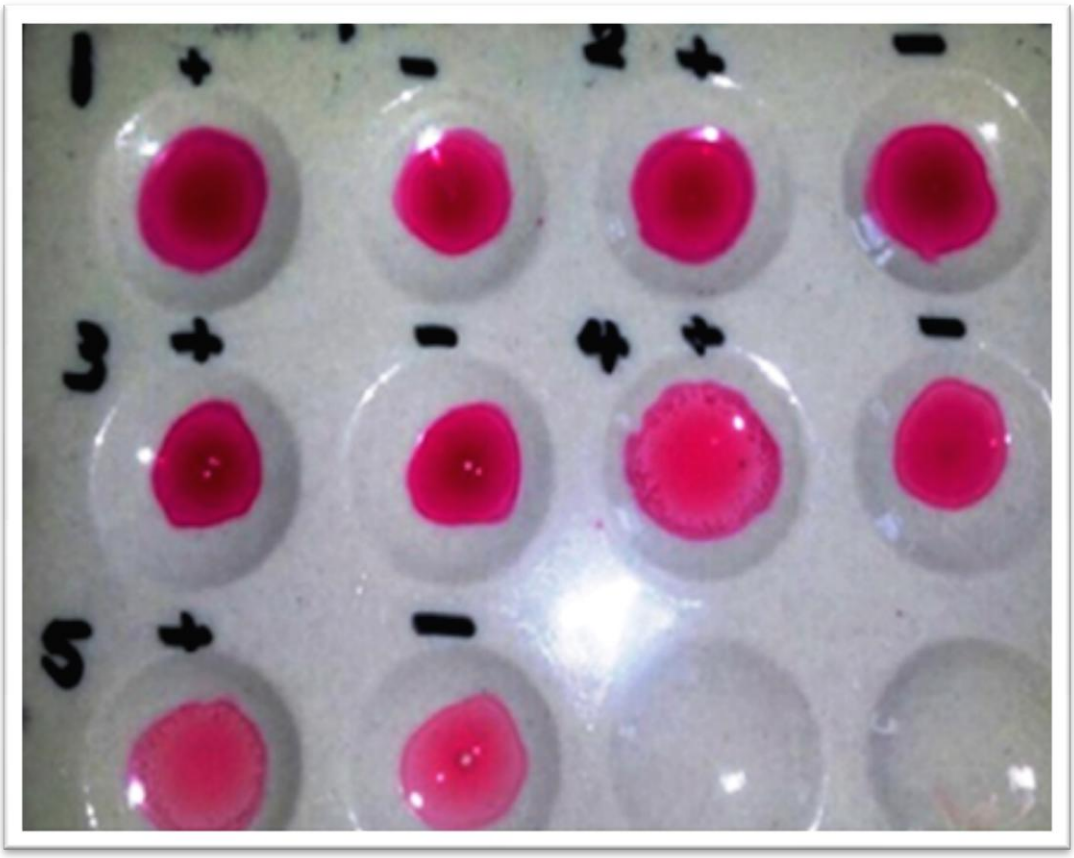

Figure 3.18.Titration after dilution of CBPP stained antigen:

1:90%, 2:70%, 3:50%: showed dense and invisible agglutination

4:30%: showed fine and visible agglutination

5:10%: not very clear agglutination.

3.3.5. Validation results with field samples (sensitivity and specificity of the developed antigens):

Reference positive and negative control sera of CBPP brought from (APHA) were used to assess the developed antigen. The results revealed typical reaction with the positive and negative sera.

All the (15) positive serum samples from the CBPP infected farm were found to be positive using both tests the serum agglutination test produced from T1/44 reference strain (SAT_1) and serum agglutination test produced from RH local strain (SAT_2) this indicated high sensitivity and specificity of the test in spite of small samples tested (Table 3.4).

Total of 222 bovine serum samples were tested with the developed antigens-from reference and local strains of *Mmm* and compared with the c. ELISA. Then specificity, sensitivity, PPV, NPV and kappa agreement tests were done to compare the concordance between individual tests. The results are shown in Table 3.5, 3.6 and 3.7. Using two by two Table layout.

Table (3.5) showed that the sensitivity of Latex test was 95% in comparison of c.ELISA and the specificity was 59.6%. Table (3.6) showed that the sensitivity of SAT_1 test is 100% in comparison of c.ELISA and the specificity was 31%. On the other hand, Table (3.7) showed that the sensitivity of SAT_2 test is 92% in comparison of c.ELISA and the specificity was 70%.

Table (3.7) and Table (3.8) showed the sensitivity and specificity of SAT_1 and SAT_2 comparing with LAT respectively. The sensitivity of SAT_1 was 98% and specificity 50%.The sensitivity of SAT_2 was 65% and specificity 82%.

Table (3.4) Seropositive serum samples from CBPP infected farm in Soba used to evaluate different serological tests

Sample No.	c. ELISA result	LAT result	SAT_1 result	SAT_2 result
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
13	+	+	+	+
15	+	+	+	+

Table (3.5): The sensitivity and specificity of LAT* comparing with c.ELISA

		ELISA		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
Latex	Positive	39 (34.8)	73 (65.2)	95	59.6	34.8	98
	Negative	2 (1.8)	108 (98.2)				
Total		41 (18.5)	181 (81.5)				

Mc Nemar Test =.001
Kappa Agreement = 0.3
Latex* =Latex agglutination test
Sen. = Sensitivity
Sp. = Specificity
PPV = Positive predictive value
NPV= Negative predictive value

Table (3.6): The sensitivity and specificity of SAT_1 comparing with c. ELISA**

		ELISA		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT1	Positive	41 (24.7)	125 (75.3)	100	31	24.7	100
	Negative	0 (0.0)	56 (100)				
Total		41 (18.5)	181 (81.5)				

Mc Nemar Test =.001
Kappa agreement = 0.1
SAT_1**=Slide agglutination test produced from T1/44 reference strain.

Table (3.7) :The sensitivity and specificity of SAT_2* comparing with c.ELISA**

		ELISA		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT2	Positive	38 (41.3%)	54 (58.7%)	92	70	41.3	97.7
	Negative	3 (2.3%)	127 (97.7%)				
Total		41 (18.5)	181 (81.5)				

Mc Nemar Test =.001
Kappa Agreement = 0.3.
SAT_2***= Slide agglutination test produced from Local strain.

Table (3.8): The sensitivity and specificity of SAT_1 comparing with LAT

		LAT		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT_1	Positive	110 (66.3)	56 (33.7)	98	50	66.2	96.4
	Negative	2 (3.6)	54 (96.4)				
Total		112 (50.5)	110 (49.5)				

Mc Nemar Test =.001
Kappa agreement = 0.4

Table (3.9): The sensitivity and specificity of SAT_2 comparing with LAT

		LAT		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT_2	Positive	73 (79.3)	19 (20.7)	65	82.7	80	70
	Negative	39 (30)	91 (70)				
Total		112 (50.5)	110 (49.5)				

Mc Nemar Test =.001
Kappa agreement = 0.4

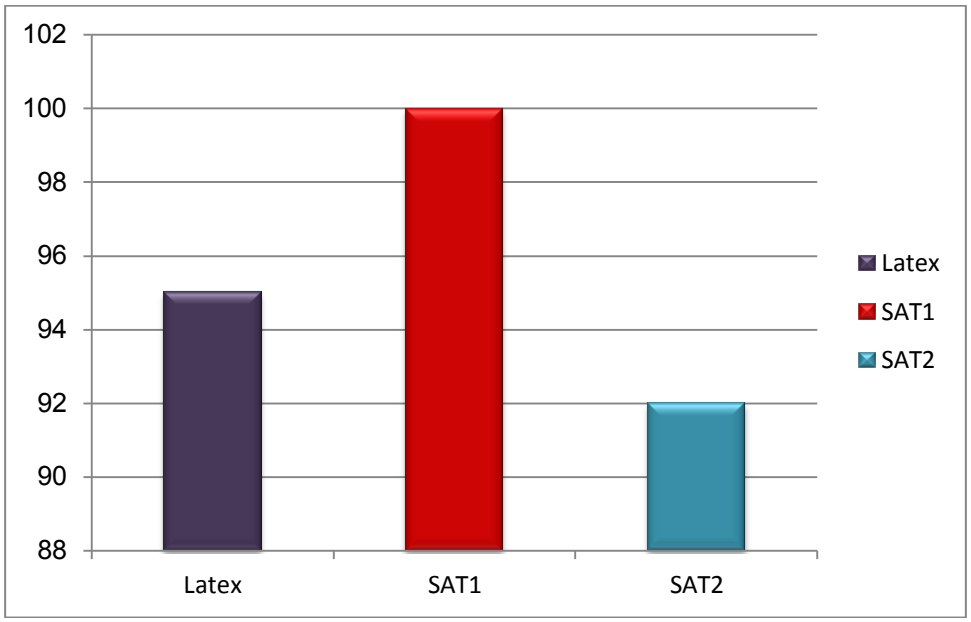

Figure 3.19. Comparison of the sensitivity of the three tests, using c ELISA as a golden test. (SAT_1 was the most sensitive test).

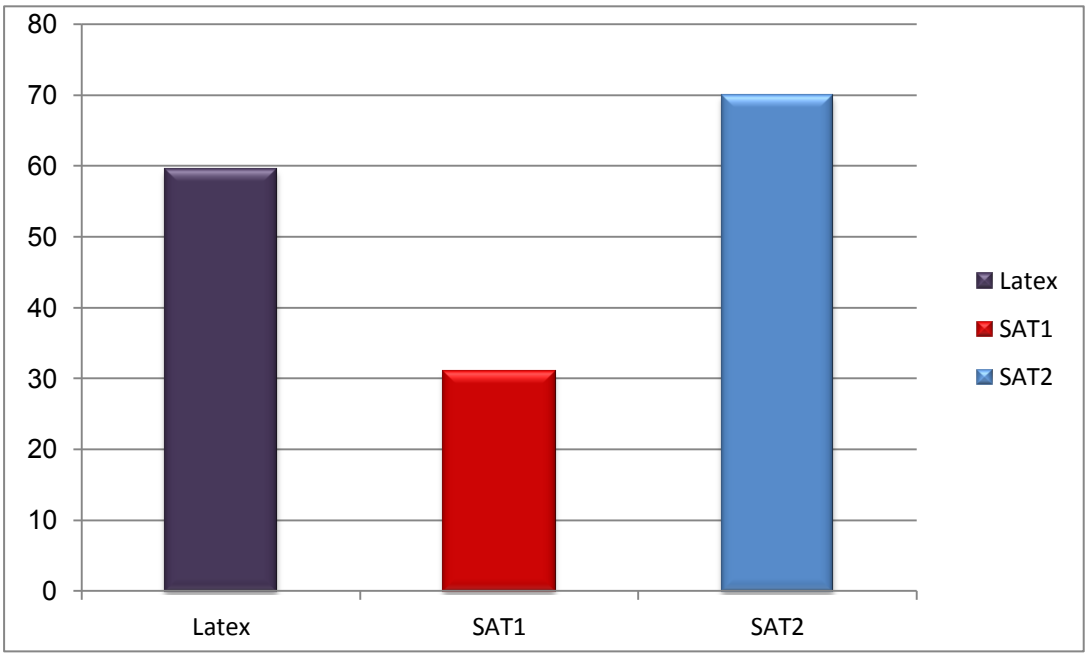

Figure 3.20. Comparison of the specificity of the three tests, using cELISA as a golden test. SAT_2 was the most specific test.

3.3.6. Antigen cost

The developed antigen reflected low cost than other trade diagnostic kits; like c ELISA and Latex test; the table below described the actual total cost.

Table (3.10). Calculation of developed antigen price \ 2ml:

Component	Total price	Quantity used	Price/unit
Bacto tryptose (Difco)	4.255.00/500g	20 gm	170.20
Yeast Extract (Oxoid)	1,20700/500g	1 gm	2,414
Glucose	69000/1kg	5gm	3,45
Sodium chloride	8200/1kg	5 gm	, 410
Horse serum (Sigma)	1958/500ml	100 ml	391,6
Rose Bengal Dye	87500/25gm	,1 gm/10 ml	3,5
Di sodium hydrogen phosphate	265,00/500g	2.5gm	1,325
Others			27.11
Total cost			**600**

One (1) liter produce (2 ml) of the antigen to test (100) serum samples.

Total Cost\ Sample: 600/100= 6 SDG this include media component, additives and others.

Latex Agglutination Test price/ sample = 250 SDG.

CHAPTER FOUR

DISSCUSSION

CBPP is one of the major infectious diseases which affecting cattle in Africa. This situation necessitates continuous surveillance and data collection in Sudan to eliminate quickly positive reactors and hence, restricting the spreading of the disease. Based on this study using serological method, CBPP was found to be an important cattle health problem in the Eastern Sudan. From economic standpoint, this area should be free from CBPP because it is considered as an export channel, with the largest market of animals and contains large dairy farms with their susceptible exogenous breeds.

A total of 1960 serum samples were tested using c.ELISA. The World Organization for Animal Health (OIE, 2008) recommends the use of complement fixation test (CFT) or competitive enzyme-linked immunosorbent assay (c.ELISA) as herd-level test. The c-ELISA may have advantages in terms of ease of testing and standardization of results, while the complement fixation test (CFT) is impractical to be used routinely on a large scale, because it needs skilled technicians for test performance and preparation of solutions. The c.ELISA has sensitivity level similar to CFT, and thus will enable the persistence of disease as seen with CFT (Le Goff and Thiaucourt, 1998). The validation tests had been performed in Africa (Thiaucourt *et al.*, 1999). In accordance with other studies, none of the present serological tests was capable of detecting all CBPP infected animals (Schubert *et.al*, 2011), due to the risk of false-positive or false-negative results in individual animals.

The different disease outcomes (acute, subacute, or chronic disease) associated with different or total absences of clinical signs hamper the diagnosis

of CBPP, and the use of more than one serological test is currently recommended Amanfu *et al*., (2000), Muuka *et al*., (2011).

In the present results the overall CBPP seroprevalence in Eastern states was found 8.2% from total samples tested. The seroprevalence was 12.2% in Al Gedaref, 6.7% in Kassala and 4.1% in Red sea states. During the year of surveillance the herds had no vaccination history so the seropositive animals were just due to infection.

In Al Gedaref state the seroprevalence was very high in the southern parts and that may due to its location at Ethiopian border, which is classified as endemic and epidemic areas as mentioned by Gedlu, (2004), Nejash and Nesradin (2017). Also Kassaye and Molla (2013), stated that the country experiences the largest number of cattle deaths, and reduction in cattle products under both endemic and epidemic conditions compared to the other African countries, due probably to its large cattle population. Generally countries in East Africa reported 66% of the total outbreaks; 58% in Ethiopia and Tanzania, and 8% in other countries in the region (Tambi *et al.,* 2006; Alemayehu *et al*., 2014).

Kassala state has borders with Al Gedaref state and Eretria and that exposed it for disease transmission during animal movement between states and neighboring countries, the highest seroprevalence was found in southern parts (Wad Al Helew locality) which has border with Al Gedaref state.

Red sea state to some extend had low seroprevalence compared with the two above states; this is due to the poor vegetation area reflected on few animal populations. In Swakin it was found that the seroprevalence was high, Swakin is a sea port in which the animals gather for exportation and that may gave chance of disease transmission.

The distribution of the disease was increasing during decades and hazard of the disease must be under focused Thus, it requires great attention both at production area and the quarantine stations. As its occurrence may cause

restriction on the trade of animals and animal products internationally, this is affecting the export earnings of the country.

In the present study an isolate of *Mmm* was recovered from dairy farm at Soba region. The animals showed typical acute clinical signs including nasal discharge, shallow and rapid respiration and cough which are typical symptoms of the disease as reported in OIE, (2018). The postmortem findings were adhesions to the chest, lung lobules showing red color and hepitization.

The isolates were identified as *Mmm* based on microscopic appearance. The broth culture was investigated by dark field microscopy; short or long filaments indicated the presence of Mycoplasma (Tully, 1983[b], Isam, 2005). The colonies were small, 1 mm in diameter; with the classical 'fried-egg' appearance were typical colony characteristics as mentioned in literature (Quinn *et al*., 1994; OIE, 2018). The isolates were inhibited by digitonin which differentiate mycoplasma from acholeplasma (Tully, 1983[c], Isam, 2005 and Ameera, 2011) based on their cholesterol dependency. The isolate was positive for glucose fermentation test and tetrazolium reduction and negative for arginine hydrolysis, this result is in agreement of Ameera (2011) and OIE (2018) manual.

The growth inhibition test (GIT) is recommended by the OIE (2018). It is simple, specific, false negative and false positive results are rare. The present result revealed a small inhibition zone around the disk as Isam, (2005) results. The small inhibitory zone can be attributed to many factors, Wallace and Clyde (1983) reported that the inhibition does not consist always of sharply defined zone surrounding antiserum disks. Strain variation and antiserum quality in addition to inoculums size and titer, affect on it; generally, any clear-cut suppression of growth can be considered as a positive result. The PCR has confirmed the previous suspicion. The PCR test approved the isolate is *Mmm* as previously described by Ameera (2011) and OIE (2018) manual. This finding supported the isolation results and gave additional evidence for the diagnosis of the disease.

Bio informatics results showed high similarity to two strains in gene-bank which confirmed that it is *Mmm* strain.

There are some limitations to estimate CBPP disease situation in Sudan like high cost of the kits and limitations to import it. The absence of rapid field test has been a hindrance to control CBPP disease in Sudan. The seroprevalence results showed that CBPP is considered an important disease in the study area tested. For that development of CBPP stained antigens -as a rapid test- from reference and local strain to contribute in CBPP diagnosis was implemented.

The antigens were subjected to the quality control tests during production; such as sterility, purity, identity and viable count (FAO, 1996). The Mycoplasma cells were stained with Rose Bengal stain as prescribed by Morgan *et al*., (1978) in Brucella antigen preparation with some modifications.

The developed antigens and Latex agglutination test (APHA-ENGLAND) were compared with c.ELISA as a golden test (IDEXX –France). Also the developed antigens were compared with LAT test. The c.ELISA has true specificity at least 99.9% and the sensitivity level is 63.8% (Le Goff and Thiaucourt, 1998).

The sensitivity of the developed stained antigen from T1/44 strain (SAT_1) was found 100% in comparison of 92% and 95% of SAT_2 (RH local strain) and LAT respectively compared with c.ELISA. March *et al*., (2002) found that the sensitivity of LAT was 62% comparing with CFT test, which is lesser than our result. This could be due to different gold standard used by these authors, as stated by Amanfu *et al*., (2000) and Muuka. *et al.*, (2011), who reported that the c.ELISA test is more sensitive than CFT in terms of sensitivity for chronic carriers but less sensitive than CFT for the detection of acute cases. Roland, (2004) stated that in a number of countries the c.ELISA detected more animals than the CFT, so the true sensitivity of the c.ELISA is considerably higher than the relative sensitivity compared to the CFT.

Priestly (1951) and Newing and Field (1953), concluded that the slide flocculation test - which has likely similar development procedure for $SAT_1$and

SAT_2- was more sensitive than that CFT and as reliable to it in chronic cases. In acute stage the Slide Agglutination Test (SAST) proved to be very sensitive and reliable as stated by Turner and Etheridge (1963).

The specificity of the developed SAT_2 antigen revealed satisfied specificity results (70%),which is higher than SAT_1 (31%) and LAT (59.6%), the high specificity percentage is due to close antigenic relationship between the local strain and antibodies circulating in the local animals so this encouraged the usage of autologous antigens in serodiagnostic assays to increase sensitivity of the tests. The sensitivity of SAT_1 was 98% and specificity 50% and the sensitivity of SAT_2 was 65% and specificity 82% compared with LAT test. This indicated that SAT_1 is more sensitive and SAT_2 is more specific comparing with LAT test. This result confirmed that SAT_2 is the test of choice to confirm positive reactors animals.

All serum samples (15) from the CBPP infected farm which confirmed by isolation of the causative agent were positive using both tests (SAT_1, SAT_2) this indicated high sensitivity and specificity of these tests (100%).

The more sensitive test, the better in the negative predictive value (NPV) and SAT_1scored the highest NPV(Pfeiffer, 2002). The same author mentioned that, the more specific a test, the better in the positive predictive value PPV and SAT_2 scored the highest PPV. If the test result is negative, the negative predictive value will be high, meaning that the animal is likely not to have the disease.

On the other hand Pfeiffer, (2002) mentioned that the diagnostic test should have at least 95% sensitivity and 75% specificity to confirm that an animal is free from disease ("rule-out"), and to study the prevalence on a certain area. The sensitivity of SAT_2 was 92% and specificity was 70%.

The stability of the developed antigen despite prolonged exposure to temperature as high as 37 ºC for 48 hours, is likely to be of great value in using it in the field. Also the antigen is stable for 12 months at 4ºC.The Latex test is also stable for 12 months at 4ºC (March *et al*., 2002).

CONCLUSION

The situation of CBPP was estimated in the Eastern part of Sudan within its localities and showed that the prevalence of the disease is high in Al Gedaref state (12%), Kassala state (6.9%) and Red sea state (4.1%).The role of open border policy between Sudan and other border countries could plays an important role in disease transmission. New CBPP stained antigens have been produced successfully from T1\44 reference and RH local strains which isolated during the study and considered an important ease and rapid control tool of CBPP diagnosis. The antigens are sensitive and a reliable test for detection animals harboring the disease (acute stage). .

RECOMMENDATIONS

This study recommended that:

1- Annual vaccination with cattle movement control through creating awareness about the disease to the communities.

2- Improvement of the public and private veterinary service delivery has a major impact in mitigating the risk imposed by CBPP.

3- Further research should be tackled to produce a purified specific antigen from local isolates to reduce the cross reaction; so the capsular polysaccharide antigen (CPS) production will help in more accurate results.

4- The search for new diagnostic tests with high sensitivity and specificity should be continued.

5- Whole genome sequence application for RH local strain should be done to show the variability of the strain from other strains in gene bank.

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APPENDIX

Appendix 1: Culture media for *Mmm* isolation*:*

Liquid or Broth media*:*

Gourlay's medium (Gourlay, 1964), (FAO, 1996):

It was consisted of the following ingredients:

Part A:

- Bacto-tryptose (Difco) 20.0 gm
- Glucose 5.0 gm
- Na chloride 5.0gm
- Disodium hydrogen phosphate 2.5gm
- Glycerol 5ml
- Yeast extract 1.0gm
- Distilled water 900 ml

Part B:

- Horse serum 100 ml
- Penicillin 100,000 IU 0.5 ml
- Thallium acetate 10% 0.25 ml
- Phenol red 0.02 gm

Solid ingredients in part A were dissolved by magnetic stirrer and pH was adjusted to 7.6. Part A was then sterilized by autoclave at 121ºC for 15 minutes. After cooling to 45-50 ºC, part B was then added aseptically to part A and the mixtures was distributed in 4.5 ml amount in to sterile test tubes. To check sterility, the medium was incubated at 37º C over night, then stored at 4º C and used within four weeks.

Appendix 2: Preparation of Solutions*:*

Penicillin solution:

This was prepared by dissolving Benzyl penicillin sodium (one million I.U in powder form) in Five ml sterile distilled water to give 200,000 I,U ml and this stored at 4°C until used within one week, or stored at -20 °C until used within one year.

Thallium acetate 10% (w/v):

This was prepared by dissolving 10 gm of thallium acetate in 100 ml of distilled water and sterilized by autoclave at 121°C for 15 minutes.

Glucose 50%

Ten percent (w\v) solution of glucose : 50gm of glucose (Merck) dissolved in 100 ml distilled water and distributed in 10 ml amounts, then sterilized by autoclave at 108°C for 5 minutes or by filtration through 0.45µm membrane filter and then stored at 4°C until used.

Yeast extracts solution 25 %(w/v):

This was prepared by adding 25 gm of yeast extract (Oxoid) in 100 ml of distilled water. Then it was distributed in 10 ml amounts and sterilized by autoclaving at 108°C for 5 minutes, then stored at -20°C until used for up to one year.

-Serum preparation for media:

Blood was collected aseptically from the jugular vein of a healthy horse in a bottle containing 20% trisodium citrate (20 ml/1000ml of blood) and kept overnight to separate plasma. One ml of 10% calcium chloride was added to one liter of plasma under mixing to separate fibrin. The serum was heated by placing in water bath at 56C for 30 minutes to destroy complement. Then sterilized by Seitz filter (0.22 micron) (14 D, EKS) and kept in the refrigerator at 4 C until used.

But on the study readymade serum (Sigma) was used.

Appendix 3: Biochemical media

Glucose Fermentation Medium (FAO, 1996):

- Heart Infusion Broth, 2.5%W/V solution

Or (Brain Heart Infusion Broth 3.7%W/V) 180.0 ml

- Horse Serum (inactivated) 40.0 ml
- Yeast Extract(25%W/V) 20.0 ml
- DNA stock solution(0.2 w/v) 2.6 ml
- Glucose (50% w/v solution) 2.0 ml
- Phenol red (1% w/v solution) 5.0 ml

The ingredients were dissolved thoroughly by magnetic stirring. The pH was adjusted to 7.8 by using 1M NaOH then sterilized by filtration through EKS 2 Seitz filter pads or autoclave at 121ºC for 15 minutes. The above mixture of ingredients was dispensed into bijoux bottles at 2.25 ml per vial and stored at 4°C. Dispensed medium was used within 30 days

Arginine Hydrolysis Medium (FAO, 1996):

- Heart Infusion Broth, 2.5%W/V solution

Or (Brain Heart Infusion Broth 3.7%W/V) 180.0 ml

- Horse Serum (inactivated) 40.0 ml
- Yeast Extract (25%W/V) 20.0 ml
- DNA stock solution (0.2 w/v) 2.6 ml
- L-Arginine monohydrochloride (30% w/v solution) 8.5 ml
- Phenol red (1% w/v solution) 5.0 ml

The ingredients were dissolved thoroughly by magnetic stirring. The pH was adjusted to 7.8 by using 1M NaOH then sterilized by filtration through EKS 2 Seitz filter pads or autoclave at 121ºC for 15 minutes. The above mixture of ingredients was dispensed into bijoux bottles at 2.25 ml per vial and stored at 4°C. Dispensed medium was used within 30 days.

Medium for Testing Reduction of Tetrazolium (FAO, 1996):

- Heart Infusion Broth, 2.5%w/v solution

Or (Brain Heart Infusion Broth 3.7%w/v) 180.0 ml

- Horse Serum (inactivated) 40.0 ml
- Yeast Extract (25%w/v) 20.0 ml
- DNA stock solution (0.2 w/v) 2.6 ml
- Triphenyltetrazolium chloride (2% w/v solution) 5.0 ml

The pH was adjusted to 7.8 by using 1M NaOH then sterilized by filtration through EKS 2 Seitz filter pads or autoclave at 121ºC for 15 minutes. The above mixture of ingredients was dispensed into bijoux bottles at 2.25 ml per vial and stored at 4°C. Dispensed medium was used within 30 days**.**

Digitonin stock solution 1.5 %(w/v):

Seventy five mg of digitonin was added to five ml of 95% ethanol in screw caped bottles. This solution was heated gently by placing the bottle in a flask of boiling water to dissolve the digitonin completely. It was then stored at 4°C until used. Filter paper disks (approximately 6 mm in dimeter) were placed in sterile plastic petridish, 0.025ml of digitionin solution was added to each disk, dried overnight at 37°C and stored at 4°C until used (Tully,1983[c]).It may be used for up to one year.

Appendix 4: Latex Agglutination Test (LAT) Reagent

- BoviLAT Latex Reagent: 2.0 ml ready-to-use antigen coated latex beads supplied in dropper bottle. Also contains sodium azide to a final concentration of 0.1%.
- Negative Control serum: Negative bovine serum.
- Positive Control: Diluted rabbit antiserum against capsular polysaccharide (CPS).
- Reaction Slides: Black cards with eight reaction cells per card.
- 20µl Disposable Droppers: 10 droppers for adding blood or serum to the reaction slide.

Appendix 5: Chemicals and Reagent for c.ELISA:

1- *Mmm* Antigen coated plate

2- Weak positive, strong positive and negative controls.

3- Conjugate concentrate

4- Dilution buffer N24

5- TMB substrate N13

6- Stop solution

7- Wash concentrate

8- Detection solution (monoclonal antibody: Mab 117/5)

Appendix 6: Buffer solution-Phosphate buffer saline (PBS):

It consisted of the following:

-Sodium hydrogen orthophosphate	1.4 g
-Sodium chloride	7.0 g
-Potassium chloride	0.2 g
-Potassium hydrogen phosphate	0.2 g
-Distilled water	1000 ml

PH adjusted to 7.3

Appendix 7: The inoculated broth culture and the *Mycoplasma* Rose bengal-stained antigen

Broth culture of *Mmm* showing turbidity and color changed from red to orange

The stained CBPP antigen (The product)