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PREPARATION OF FREEZE DRIED *CLOSTRIDUM CHAUVOEI* MASTER SEED BANK FROM LOCAL ISOLATE FOR PRODUCTION OF SAFE, EFFECTIVE AND ACCEPTABLE QUALITY OF BLACK LEG VACCINE

MSC research

By:

JALETA SHUKA

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENTOF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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PREPARATION OF FREEZE DRIED *CLOSTRIDUM CHAUVOEI* MASTER SEED BANK FROM LOCAL ISOLATE FOR PRODUCTION OF SAFE, EFFECTIVE AND ACCEPTABLE QUALITY OF BLACK LEG VACCINE

A thesis submitted to college of veterinary medicine and agriculture of Addis Ababa University in the partial fulfillment of the requirements for the degree of Master Science in veterinary microbiology

By:

JALETA SHUKA

Advisor:

Hika Waktole (Assistant Professor)

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Approval sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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Title: Preparation of Freeze-Dried *Clostridium Chauvoei* Master Seed Bank from Local Isolate for Production of Safe, Effective and Acceptable Quality of Black Leg Vaccine

Submitted by: Jaleta Shuka _____ _____

Name of student Signature Date

Approved for submittal to MVSc research thesis assessment committee:

1. Hika Waktole (MSc, Assistance prof.) _____ _____

Principal advisor Signature Date

2 Gezahagne Mamo (Proffesor) _____ _____

Departiment Chairperson Signature Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Jaleta Shuka Entitled "preparation of freeze-dried *clostridium chauvoei* master seed bank from local isolate for production of safe, effective and acceptable quality of black leg vaccine" and recommend that it is accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

_____	_____	_____
Chairman	Signature	Date
_____	_____	_____
External Examiner	Signature	Date
_____	_____	_____
Internal Examiner	Signature	Date

Advisors

2 Hika Waktole (Assistance prof.)	_____	_____
Principal advisor	Signature	Date

STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

CLSI	Clinical and lessons Standard Institute
CSA	Central Statistical Agency
CSY	Casein Sucrose Yeast
EA	Environmental Agency
FAO	Food and Agricultural Organization
FAT	Florescence Antibody Test
GIS	Geographic Information System
ISO	International Office Standardization
IHC	Immunohistochemistry
IM	Intra-Muscular
IV	Intra-Venus
LD-50	Lethal Dose-50
MM	Millimeter
NMSA	National Meteorology Service Agency
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
QMS	Quality Management System
RBC	Red Blood Cell
SDS	Sodium Dodecyl Sulfate
TEM	Transport Enrichment Medium
USA	United State of America
UV	Ultra Violet
VF	Viande-foie
WBC	White Blood Cell

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ABSTRACT

The research was accompanied from December 2020 to May 2021 amid to develop new preservation method spore form of *Cl. Chauvoei* from local isolate with the objective of mainly improving vaccine immunogenicity. Currently, in National Veterinary Institute (NVI) of Ethiopia viande-foie (V.F) medium is the sole medium used for preservation of the strain as a vaccine master seed bank. However, this method of preservation has its own drawbacks like low antigenicity maintenance, prone to contamination and difficulty in transportation. Intended for this purpose, samples were composed from Adea district from cattle following outbreak information of black leg disease. The samples were taken to NVI using cold chain system for isolation purpose. The blood and tissue samples were isolated as *Cl. Chauvoei.* The isolates were identified by cultural, morphological and biochemical features. With the aim of additional confirmation by means of pathogenicity study and acuity estimation, the isolates were inoculated on gunia pigs and up on postmortem examination it was confirmed as *Cl. Chauvoei*. The study of PCR also revealed the existence of *Cl. Chauvoei.* New method of preservation was then developed through lyophilization or freeze-drying of the isolated strain. Quality control tests like vacuum, moister content, viability, sterility, safety, lethal dose determination for potency test, and identity tests of freeze-dried spore form of *Cl. Chauvoei* seed bank were performed. Accordingly, the quality control tests of the freeze-dried product revealed; vacuum (1.5%), moister content (97.5%), LD50/ML ($1 \times 10^{7.2}$), known to be viable, safe and free of contaminant. Regarding potency test 10 gunia pigs were challenged with LD50 dose of virulent challenge strain, and only one of them was dead, thus it is 90 % protective. It is believed that freeze-dried spore stored at -20^{0} C retain viability for years, however in this specific study stability test at different storage temperatures for long period of time is essential to conclude this idea. Moreover, study reinforced by DNA sequencing is also indispensable to finalize these inferences.

Key words: *Freeze-drying, Glyceration. Preservation, Vaccine.*

1. INTRODUCTION

Ethiopia is amongst nations with the largest domestic animals in Africa and the area has a principal impact in the development of economy from the farming industry. The ruminant animals' population in Ethiopia by type is believed to be cows (59 million), sheep (30, 697 million), and goats (30, 200 million) (CSA, 2017).

However, the progress of the segment in Ethiopia is delayed by great substantial future health constraints counting bacteriological infections, virus-related syndromes, and parasitic invasion (Yune and Abdela, 2017). Among these one of bacterial pathogenic microorganism called black leg is acquired by one of clostridia bacterial animal pathogen namely *Cl. Chauvoei.* Yet, there is a believe that *Cl. Speticum* can also the probable source of this illness, as there is proof of frequent isolation of the strain as of black leg lesions (OIE, 2012). Owing to this reality, few of global vaccines producing company prepare bivalent vaccines consisting of both *Cl. Chauvoei* and *Cl. Speticum* strains which are used as control and prevention. On the contrary, lack of isolation of *Cl. Speticum* from original samples of dead animals with black leg makes doubt if this unique bacterium is the preliminary cause of the disease or after death invader (OIE, 2012). Because of this fact *Cl. Chauvoei* is the only strain used for the production vaccine against black leg disease in most vaccine producing laboratories around the globe including NVI of Ethiopia.

The organism causing black leg is one of Clostridial species identified as to be motile, anaerobic, staining gram positive that affect both cattle and sheep and is related to histotoxic group (Bagge *et al.,* 2009). The organism is understood to be the most existing pathogen resulting in high death specially in cattle (Smith and Williams, 1984). The bacteria usually occur in alimentary tract of those ruminant animals (Habib *et al.,* 2015). It is known that the spore form of the bacteria exists in paramount in the soil in every place affecting grazing animals. The effect of the disease is facilitated once the cattle intake the spore, and endospore then penetrates the intestinal mucosa to get in to the circulation to be stayed in the animal physical particularly in skeletal system. In the skeletal muscle the

germination of the spore is facilitated in certain conditions like injury and extreme exercise that provide optimum condition for its growth. Then, the vegetative bacteria start to proliferate, ferment muscle glycogen, breakdown protein and yield foam and toxins (Richey, 2004). Situations favoring for germinations of spores in the body, growth of the organism, and proliferation of toxins result in confined lesions such as organ bleeding, oedema and muscular necrosis (Inam-ul-Haq and Niamatullah, 2011).

Consistent with Chandler and Gulasekaram (1999), there is no prevalence of assortments between varied immunotypes of *Cl. Chauvoei*, though the Australian one named as CH3 is found to be more potent. Despite the fact that there is a suitable degree of cross defense between the two strains *Cl. Chauvoei* and *Cl. Speticum*, a few studies discovered that there is a moderate difference (Kerry, 2010). In early years in Australia after vaccination of livestock with vaccine made out of *Cl. Chauvoei* breakdown of immunity has been conveyed, as a result others strains had been mixed in to the vaccine to increase the advantage of maximum degree of booster (Reed and Reynolds, 2008).

Control and prevention of the disease mainly based on vaccination. Using well known registered seed bank strain that able to give safe and immunogenic protection against this specified disease is very crucial, thus for production of vaccines using such kind of strain is very important (British pharmacopeia, 1985). Consequently, to have such vicinal seed strain it's far very crucial to validate the preservation method of *Cl. Chauvoei'* (Karber, 1998).

As discussed earlier the vacinal seed bank used for black leg vaccine production must be a well characterized with recognized origin. However, local from region might be used. It's far beneficial not to use original isolate of *Cl. Chauvoei*, as an alternative it must be first investigated for its suitability, then preserving the antigensity through propagating in life animals, properly cultivating and preserving could be very critical. The preservation methods can be achieved by two approaches namely: using VF medium or through freeze drying. Furthermore, freeze drying is divided in to two forms consisting of preservation through Lyophilization of the vegetative and spore form, and for the best of

immunogensity and longtime preservation it is essential to use freeze-drying of spores (Henry and Stampfli, 2014).

Preparation of master seed bank from freeze drying spores provides high duration of viability, low risk of contamination and easy transportation (Henry and Stampfli, 2014). However, in NVI of Ethiopia, only VF media is used for preservation of this specific strain which possesses high risk of contamination, difficulty in transportation and low antigensity maintenance or short duration of viability.

Therefore, the general objective of the study is to improve the level of efficiency of black leg vaccine produced at NVI by developing and using freeze dried spore form of *Cl. Chauvoei* through invitro and in vivo studies from filed isolates.

The specific objectives of the study are:

1. Improving the quality of black leg vaccine produced at NVI through development of best preservation method of the strain used as master seed bank. And
2. To make well known registered strain available at NVI

2. LITRETURE REVIEW

Overview of black leg Disease-

Black leg disease is a non-contagious infectious disease caused by *Cl. Chauvoei* which is one of the most important clostridial microbes infecting cattle and rely sheep. Some scholars named the disease in many ways namely; quarter evil, symptomatic anthrax, black quarter, quarter ill or emphysematous gangrene (Smith and Williams, 1984). *Cl. Chauvoei* was first invented in 1887 and was late termed once a French veterinary scholar JBA Chauveau (Smith and Williams, 1984). The bacteria can exist in cluster and occasionally in chains, but in most case, it is present in a single form. Eventhogh, literally it is known to exist in a gram-positive form the grams staining features are varied and specially in very old growth, the bacteria can be seen as gram negative under microscope. The existence of flagella enables the bacteria to be motile. The organism can sporulated in very unfavorable environment, developing ovoid, and mostly sub-station spores. Chemicals like chlorine, iodine and oxidizing products can readily abolish even the sporulated types of the bacteria. However, the action of phenolic and quaternary ammonium can be tolerated by the sporulated bacteria (Abreu *et al.,* 2016). It produces colonies which are rounded and bounded by a thin band on blood agar. However, the futures of the colonies morphology depend on the species from which the blood (RBC) is collected and the strain of the bacteria (Popoff, 2016).

Animals acquire the pathogen during grazing by ingesting the contaminated feed with the spore from the organism. In the animal body with in the small intestine the bacteria make attachment in to the epithelial layers, and get in to the blood stream by pricing it. In the musculoskeletal system the spore remains latent until it gets favorable conditions for which facilitate its growth. Less oxygen environment created due to muscle damage favors the germination of the spores**,** then after-multiplication toxin could be produced that's accompanied by hemorrhage and necrosis of muscle (Cooper and Valentine, 2016). *Cl. chauvoei* is started in oxygen scarcity atmosphere like injured fleshy tissue. Conditions poor handling can result to tissue harm that consists of blood circulation, subsequenting in

reduced oxygenated blood distribution to that area. Although black leg is age dependent disease rarely, the disease can be observed in immunosuppressed mature cattle. In animals infected with black leg enlargement of body parts like leg, neck, hip, chest, shoulder is the furthermost shared sign of blackleg observed in lifeless animals. The inflammation is as a result of liquid and gas deposit created by the anaerobic microbes in the muscle. The pretentious parts, gas can frequently be touched moving while creating a crepitant sound beneath the skin. Wet or rainy season is the most appropriate time to the occurrence of black leg disease affecting grazing cattle. Animals with good body condition and well-nourished from the age of six months to two years are commonly affected (Knight and Kent, 2006).

The organism causing black leg is known to produce toxins that result in toxemia and finally death. The prominent clinical signs are observed staring from twelve to forty-eight hours post infection, but in a rare case it can remain silent. Whenever the clinical signs exist the most common sings appreciated are malaise, tiredness, loss of appetite, rumen inertia tachycardia and crepitation sound at affected muscle (Cooper and Valentine, 2016).

2.1. Ethology

Black leg causing bacterium *Cl. Chauvoei* is morphologically usually pleomorphic, a gram negative, very pathogenic, which produces lemon like endospore and consume enrichment media for growth purpose (Quinn *et al.,* 2011). This organism can persist with in the soil for many years due to spore forming nature, and this spore makes the organism to be proof against environmental variations and the organisms are usually pleomorphic (Falquet *et al.,* 2013). Defecation is the route by which the organism is excreted from the intestine of animals to external environment. The eruption of the disease takes place within the farm in cattle whenever there's existence of recent excavations or next to overflowing (walker and batty, 2013).

2.2. Epidemiology

Black leg is a worldwide disease in occurrence, and the organism causing the disease is considered as a soil born infection even if cannot grow in the soil. The soil is contaminated when the infected animals discharge the grown organism from the digestive tract during defecation. The organism in the external environment produces spores that enable to persist for many years by tolerating any environmental variations (Radostits *et al.*, 1994).

The prevalence of black leg in Ethiopia is almost everywhere except part of Somalia and Gambella. In most case Oromia and Amhara were the most affect area in the country. In general, blackleg is a prevailing disease in Ethiopia and can be considered one of the diseases affecting the livestock industry in the country (Tesfaye and Salehi, 2018). Following the eruption of the disease it is very crucial to do ring vaccination to the group of animals in and around outbreak area, but concerning the case morbidity and mortality no study yet completed. Nonetheless, the disease is very prominent to Dega followed by Weinadaga and Kola (Tolera *et al.*, 2019).

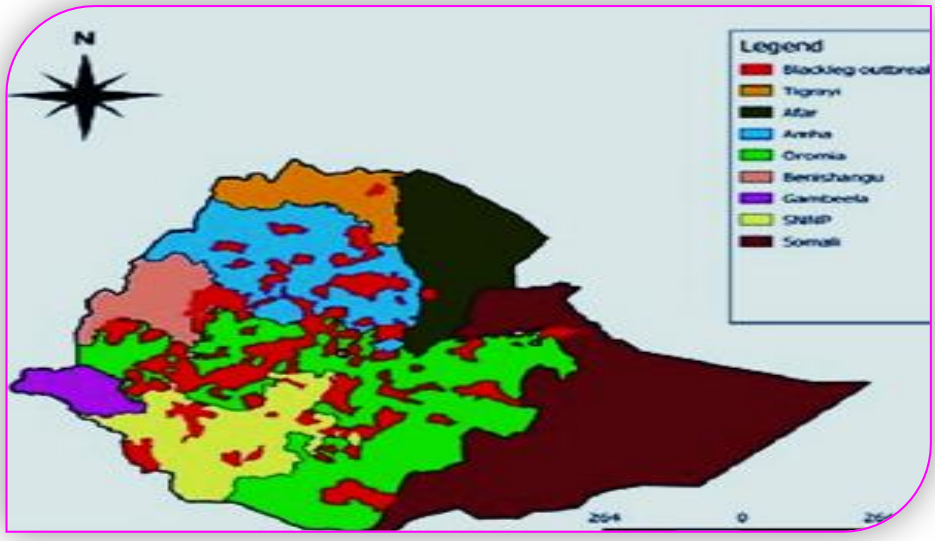


Figure 1: Diagram showing circulation of black leg disease across Ethiopia
Source: (Tesfaye and Salehi, 2018).

2.3. Risk Factors

There are a number of risk factors associated with black leg disease. These include species, breed and age of animals including environmental factor. Regarding the animal risk factor cattle and rarely sheep are those animals infected by the true blackqurter but the illness has been conveyed in deer and in single event in a horse. It is revealed that young cattle between six months to two years are commonly infected and is mostly seen in the field in fast growing cattle with good nourishment. In the case of sheep exposure to feed supplemented with high protein content will surge their vulnerability to blackleg. Unlike that of cattle in sheep all ages are at the same risk (Andrews *et al.,* 1992). Exotic breeds are also more sensitive to the disease than local breeds (Tesfaye and Salehi, 2018).

On the other hand, considering environmental risk factor the eruption of the disease is highly facilitated by wet season commonly from spring to autumn. Contingent perhaps once young animals reach the vulnerable stage some eruption of blackleg in cattle have happened subsequent to digging of soil which hints that disruption in soil might bare and trigger dormant spores (Schipper, 2008).

2.4. Pathogenesis

The disease process of *Cl. chauvoei* is associated to a number of virulence factors and toxins (Table 1). The virulence factors include toxin A (CctA), a β-barrel, pore-forming hemolysin. The CctA cause a polymer pore that make a hole in the cell membrane, causing in disturbance of membrane penetrability and cell death. Innovative trial vaccines covering CctA prove defensive immunity in vaccinated guinea pigs (Frey, 2012).

The animals ingest the bacteria from the soil while grazing the posture, and then the bacteria make attachment to epithelial layer of alimentary tract. With the aid of the blood circulation, it reaches to the muscle where it remains inactive in the form of mononuclear phagocytic structure (Quinn *et al.,* 2012). The spore can remain in the muscle in a latent form for years unless it gets favorable environment which facilitate its multiplication

(Barros, 2016). Damage to tissue facilitate anaerobic environment which favors growth and releases of toxins that result in necrosis of vascular endothelium and myofibers. Finally, the toxins will be distributed though out the body via blood circulations resulting in sever diseases and death (Useh *et al.*, 2016). Though the presently putative disease process of blackleg seems reasonable, furthermost indication to sustenance the suggested sequence of proceedings in the pathogenesis is incidental with no conclusive supportive evidence obtainable. Moreover, subjective indication proposes that bags of blackleg might to happen in cattle not exposed to injury, for which a systematic clarification is not presently obtainable (Weatherhead and Tweardy, 2012). The bacteria form toxins like alpha, deoxyribonucleic and hyaluronidase (Hagan and Bruner's, 2009). The antigens of *Cl. Chauvoei* encompass flagella, spore and somatic antigens. Many strains of clostridium species component the same somatic, spore and flagella antigens (Gyles *et al.*, 2004). The antigenicity of *Cl. Chauvoei* is varied, though there is substantial cross defense between strains (Carter and Chengappa, 1999).

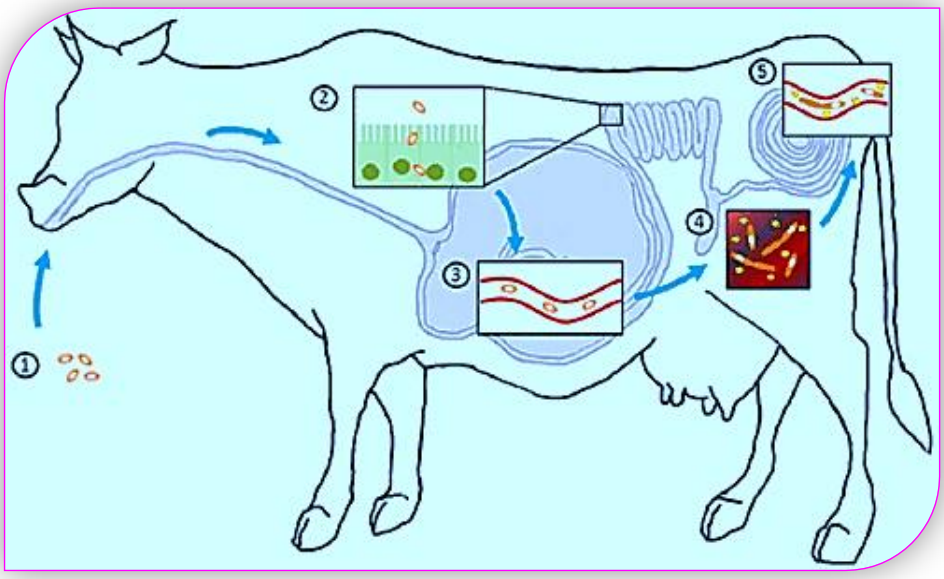


Figure 2: Studied disease process of blackleg

Source: (Useh *et al.*, 2016).

The above picture shows that the bacteria get in to the animal body while the animals are feeding pasture with contaminated soil (1) and within the body attached to the gut penetrating the intestinal layer to get in to the circulation (2). Then, first it spreads to skeletal muscle to persist there in inactive form (3). The microorganism yields exotoxins that result in confined necrosis and tenderness (4). On the quit, multiplying microbe and their toxins come into the flow instigating exotoxemia and ultimately pass forever (5).

2.5. Postmortem finding

Animals with good body condition due to feed with good dietetic form are very vulnerable to black leg (Snider and Stern, 2021). When the exterior layer of muscles is pretentious, the covering skin is stressed by causal inflammation and result in black color to the surrounding muscle. The gas content of the hypodermic tissue results in crepitant sound which is detectable upon palpation. Typical hemorrhage with necrosis and gaseous muscular inflammation is observable in skeletal systems, mainly in hind limbs, providing the area dark color, as of which the term black leg is derived. Similarly, the diaphragm and tongue are also affected and are regularly inspected for the lesions of black leg (Sinder and Stern, 2021). The lesion in these areas display different size with black or redish-balck containing numerous minor fissures formed by air deposited (Figure 3) (Frey and Falquet, 2015). Lesions in the diaphragm and tongue are comparable to those lesions observed in other muscle even if it is less serious. No gas formation is observed in the tongue part. It is believed that no lesion is observed in coronary system but one scholar from USA with unpublished writer revealed that, conflicting to preceding views, an indication of lesions in the coronary system are observed in the sever cases of black leg (Robinson and Robin, 2016). The heart lesions are mostly described as inflammation of heart muscle with hemorrhagic with necrosis formation (Figure 3) (Zachary, 2012). In heart lesions usually no emphysema that characteristically differentiate from the skeletal muscle inflammation however, there can be merely a minor volume of gaseous existence. Inflammation of pericardium is a typical non microscopic finding (Helman, 2007). In general, the postmortem finding can be described as gross and microscopic lesion as follows:

Gross lesion- the gross appreance of the infected muscle is determined by the length of the lesion. During the early phase of the infection the margin of the lesion seems pale and prominently bulged with the mixture of serum and hemorrhagic which may burst at the last (Willian *et al.,* 2014). As time of the infection proceed the lesion appreance will be changed to desiccated reddish black, permeable as of air foams. The edge of the infected muscle lesion becomes dark red, dry, necrotized tissue filled with a tiny foam which can be clearly visible (Buxton and Fraser, 2009).

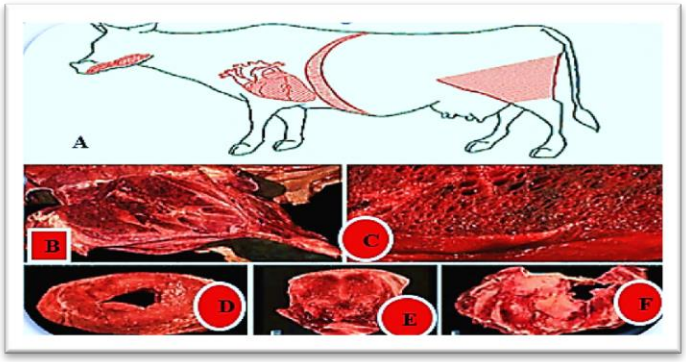


Figure 3: Non-microscopic lesions of blackqurter in cattle

Source: (Abreu *et al.,* 2016)

Letter A denotes the common anatomic area of cattle infected by *Cl. Chouvae* cases, B represents typical infection by the bacteria viewing inflamed muscle in the hind limb. Similarly, C symbolizes swollen skeletal muscle with noticeable gas contented, D signifies inflamed muscle of heart and fibrinous pericarditis. Letter E represents dead part and swollen ventral side of the tongue, and finally F symbolizes swollen diaphragm muscle with rib attachments.

Microscopic lesion-using microscope the inflamed coronary heart muscle and thymus is seen as visible sized necrotizing and suppurative lesions. In musculoskeletal system it's incredibly favored as tangent foam formation (Crichton and Valdes, 2016). Tough regions of necrosis and collections of neutrophils and lymphocytes along the muscle septal also are commonplace, but oedema is uncommon (Jordan, 2009). Up on histological pathology investigation of tissues pretentious with the disease proven necrotizing gas formation and fibrinous pleuritis (Russell *et al.,* 2009).

The histology of *Cl. Chouvae* in heart and muscle are very similar (Figure 4). With low objective under microscope, huge unfilled vacuoles, showing gas formed areas due to the accumulation of foams, commonly are seen in skeletal muscle nevertheless, infrequently in muscle of the heart (Harwood, 2007). Muscle cell demonstrate deteriorating or necrotic deviations containing of distended, vacuum, and disjointed muscle filaments. White blood cell (WBC) penetration is not a protuberant feature in this disease due to the severe nature of black leg (Sasaki, 2000). Blood vessels vicissitudes are more predominant in heart lesions. Huge figures of rod shaped, that might comprise at the hind, middle, or, infrequently, terminal spores, are existing regularly adjacent to the damaged vascular and within muscular fibers (Sasaki, 2002).

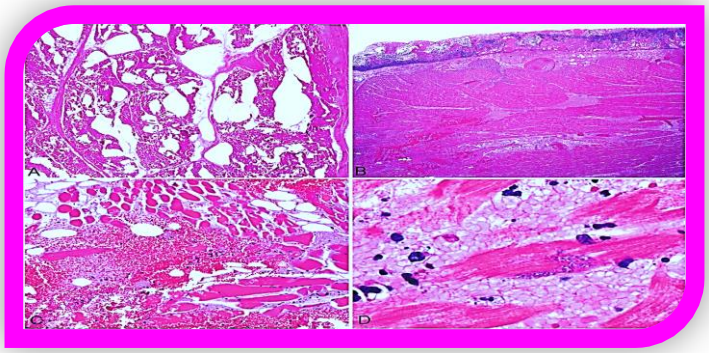


Figure 4: Histopathologic morphology as a result of *Cl. Chouvae* in cattle

Source: (Cooper *et al.,* 2016)

In the overhead picture A exemplifies musculoskeletal part with bulky cavities filled by gas, whereas B represents fibrinous epicarditis and myocardial necrosis and outflow. Similarly, letter C symbolizes sample from tongue with necrosis, and insignificant irritation. Finally, D denotes advanced exaggeration of the myocardium viewing numerous clostridial.

2.6. Diagnosis

Tentative diagnosis can be executed by means of clinical symptoms but, may be overlooked by *Cl. Speticum*, *Cl. novyi*, *Cl. sordellii*, and *Cl. perfringens*. Thus, confirmatory diagnosis is crucial using cultural and biochemical approach by taking samples from muscle, blood, liver and spleen immediately post death. PCR is known to be excellent for clinical sample but not conservational samples (Cooper *et al.,* 2016). Detection of immunity approaches, such as florescent antibody test (FAT) and immunohistochemistry (IHC) are also others methods of diagnosis(Quinn, 2011). Choices on which examinations to custom are habitually constructed on obtainability at local analytic laboratories. The main differential finding for blackleg disease is other diseases which are caused by numerous clostridial pathogens, counting *Cl. Speticum, Cl. Chauvoei, Cl. perfringens, Cl. sordellii*, and *Cl. novyi* (Quinn, 2011).

2.6.1. Clinical signs

It is common to see sudden existence of the case at the beginning in black leg, but rarely can occur deadly without showing predictive clinical signs. The first symptoms expressed is a raise in body temperature, however in the course of infection when the clinical signs are indicative the temperature tends to be normal or sometimes under. The predominant clinical signs include lameness, fever, and acute, patent depression. Typical edematous and crepitate sound develop at the inflammations in the hip and back area (FAO, 2008).

2.6.2. Isolation and Cultural method

Either of the three samples which include edematous fluid, blood from the heart or grinded muscle tissue in mortal is cultured on liver Extract-Blood agar (Oxford, England) then incubated at 37 ^{0}C for twenty-four to seventy-two hours in anaerobic environment. Finally, the morphologic characteristic of colonies is studied and the colonies with identical morphology with that of *Cl. Chauvoei* sub cultured. At the same times VF medium will be used and pure culture will be subculture on blood agar plates under anaerobic condition (Williams *et., al* 2014). Aseptically collected sample by Pasteur pipettes from muscle

tissue, heart blood, and oedema of muscle using are used for laboratory analysis (Radostits *et al.,* 1994).

2.6.3. Molecular method

Nucleic acid extension of a precise mark region of the bacterial genome by the polymerase chain reaction has remained extensively used for finding and analysis determinations. Basically, there are three types of genes to be detected during PCR method of *Cl. Chouvae*. These include detection of gene responsible for production of flagella, toxins and 16S-R (Crichton *et al.,* 2006).

PCR through primers intended for flagellin gene (fliC)-

Cl. Chauvoei flagellin prerequisite special emphasis for molecular detection of this pathogen. The purpose of flagella is for motility and it will not directly be participated in black leg disease establishing development (Stevenson *et al.,* 2015). Nevertheless, they can help the disease process as they allow the movement of the pathogen to attack the target area. In overall flagella is known to be immune apparent protein of *Cl. Chouvae* which have defensive part and diagnostic future (Usharani *et al.,* 2015). There are primers designed for flagellin gene which are specific to *Cl. Chauvoei* and *Cl. Speticum* with molecular size of 798 bp and 594 bp, respectively. Both species have shared forward primer, but with dissimilar reverse primers ((Sasaki, 2000).

2.7. Treatment, Prevention and Control

Treatment is usually unrewarding due to the rapidity of the spread of the disease, but it is determined that penicillin is the remedy of prime for treatment (Lambert, 2010). Control and prevention of the disease is possible by vaccination which is the vital scheme and bivalent vaccine containing *Cl. Chauvoei*, *Cl. Speticum* is safe and reliable for cattle and sheep. Basically the word vaccine comes from Vacca meaning cow which was the ancient name, was initially named by Edward Jenner during his experimental investigation to vaccinate human small pox virus by inoculating human with cow pox virus (Lambert, 2005). Throughout his investigation he also showed that there is a tie link among animal and human transferable viruses. In current medicinal science vaccine can be articulated as

any product formulated to enhance active immunization against specific diseases (OIE, 2012). Due to an advance of knowledge and recent technology in most case all including black leg vaccines are produced under strict quality control environments by trustworthy manufactures. However, still today there are reports establish about the existence of vaccine failure for some reasons (Sarah and Wilson, 2017). In most cases the failurity of the vaccine could be due to deficient number of antigens in the vaccine (Chakraborti, 2012).

Though, conservative black leg vaccines are prepared from the bacteria causing black leg itself formulated from formaldehyde inactivated cultures of *Cl. Chauvoei*. There is also polyvalent form of the vaccine prepared with other clostridium strains. The effectiveness and efficiency of the vaccines can be examined by the amount of antibody titer produced in immunized animals. It is revealed that the vaccines from *Cl. chauvoei* are almost fully protective in natural contact however, in experimental trial it is 50-100 percent. Since the spread of black leg is wide, primarily immunization against the disease is frequently advisable at two months of age and the second vaccination will be four to six weeks late. Yearly, boosters are advised after that, up two years of age (Sarah and Wilson, 2017).

The protective capacity of recombinant vaccines prepared from toxin named CctA were challenged on laboratory animals with infectious stain of *Cl. chauvoei*, and the result were a capable contender for innovative vaccines counter to blackleg disease. Additionally, others toxins like neuraminidase in combination with others amino acids derivative from others toxins originated from the bacteria will have maximum defensive outcome counter to blackleg. Similarly, flagella have a potential antigenic effect by enabling the bacteria to migrate with in the body (Sarah and Wilson, 2017).

2.8. Black leg vaccine production

For the production of blackleg vaccine, a well characterized strain of *Cl. chauvoei* obtained from a known source should be used. However, a local isolate from a particular area or country can also be used. Before using a fresh isolate, it is essential that its suitability be investigated. For the maintenance of antigenicity of the *Cl. chauvoei* strain, it is essential

that it should be passaged in animals, properly cultivated and preserved. The methods of cultivation, isolation, identification and preservation are described.

2.8.1. Cultivation

Anaerobic organisms do not grow in the presence of oxygen. For the cultivation of anaerobic organisms on the surface of media, oxygen must be removed from the atmosphere either by using it for combustion or by replacing it with an inert gas. A suitable anaerobic jar is used for this purpose and a mixture of 90 percent hydrogen and 10 percent carbon dioxide is ideal to create anaerobic conditions. The Gaspak system is also useful for cultivation of anaerobes.

Special media are also available for the routine cultivation of anaerobic organisms in liquid media particularly for production of vaccines. Sodium thioglycolate or L-cysteine hydrochloride are incorporated in these to reduce oxygen potential and to provide suitable anaerobic conditions. A small amount of agar in media also helps development of anaerobic conditions.

2.8.2. Isolation

Immediately after death, *Cl. chauvoei* can be isolated in pure cultures from heart blood, liver or spleen. In addition, organisms can be also isolated from the lesion at the site of the inoculation. Clean the surface of infected tissue with rectified spirit, make an incision in the center of the lesion by a sterile scalpel and remove a small portion of skin. Sear the exposed portion of the tissue with a hot spatula and aspirate the edematous fluid by inserting a Pasteur pipette. Remove a piece of muscle from the periphery of the lesion. Although cultural methods are adequate for the isolation of Cl. chauvoei, in combination with biological method they give a higher recovery rate. In cultural method Inoculate liver extract-blood agar plates with edematous fluid or blood or pieces of tissue ground in mortar, grow under anaerobic atmosphere in an anaerobic jar at 37°C for two to three days, and examine the colonies and subculture typical of Cl. chauvoei colonies. Isolation can also be done directly in an anaerobic broth medium and pure cultures obtained by subculturing on blood agar plates under anaerobic conditions.

In the case of biological method, the guinea pig is most susceptible and commonly used as an experimental animal. Grind the tissue in mortar and prepare a 10 percent suspension in physiological saline solution, and inoculate 0.5 ml by intramuscular route in the leg. Within 14 hours after inoculation, the guinea pig becomes dull, depressed, and a soft swelling develops at the site of the inoculation. After 24 hours inflammation spreads to the neighboring muscular area which fills with gas. The animal dies within 48 hours. Upon incision tissues are hemorrhagic, edematous and fluid oozes out. Muscle and subcutaneous tissues are filled with gas. Isolate the organisms immediately after death from heart blood, spleen, liver, edematous fluid and tissues from the lesion.

2.8.3. Identification

Identification of *Cl. chauvoei* can be carried out by morphological, cultural, biological and fluorescent antibody tests.The differentiation of *Cl. chauvoei* and *Cl. Speticum* is often necessary. These two are similar morphologically, and both are pleomorphic. *Cl. chauvoei* is shorter, plumper and more ovoid than *Cl. Speticum*, the former grows singly, whereas the latter frequently forms long chains. The impression smears prepared from the peritoneal surface of the liver of a guinea pig inoculated with *Cl. chauvoei* reveals citron, short forms which occur singly. On the other hand, with *Cl. Speticum* elongated filaments are observed.

Cl. chauvoei is a more fastidious organism than *Cl. Speticum* and to ensure growth a special medium containing liver extract and glucose is required. The majority of the colonies of *Cl. chauvoei* when grown on 1.8 percent agar are umbonate with raised lips, whereas the majority of *Cl. Speticum* colonies are usually spreading rhizoidal types. *Cl. chauvoei* ferments sucrose but usually not salicin and *Cl. Speticum* ferments salicin but usually not sucrose. Both *Cl. chauvoei* and *Cl. Speticum* can be readily differentiated by fluorescent antibody tests.

2.8.4. Propagation of strain in animals

The Guinea pig is an excellent laboratory model of *Cl. chauvoei* and is used for the propagation of the strain. However, it is essential to passage the strain periodically either

in sheep or also cattle. Guinea pigs weighing 300 to 400 g and one-year-old susceptible healthy cattle and sheep are suitable. Inoculate guinea pigs with 0.2 ml, and sheep and cattle with 1 ml of suitable dilution of virulent organisms by intramuscular route. When spores are used, activate by diluting in solution of 2 percent of $CaCl_2$. $2H_2O$. Inoculated animals show edematous swelling at the site of inoculation. Cattle and sheep die within four to five days and guinea pigs within two days after inoculation. Collect heart blood of the animals preferably when moribund or immediately after the death under aseptic conditions using a pasteur pipette. Isolate the organisms by seeding on the media. Organisms can also be isolated in pure cultures from edematous fluid, tissues from site of inoculation, spleen and liver of the animals.

2.8.5. Preservation method

This can be achieved by preserving Vegetative organisms or Spores. The anaerobic environment of the preserving can be maintained by glyceration means. *Cl. chauvoei* spores are best preserved by freeze drying.

3. MATERIALS AND METHOD

3.1. Research Area

The research was performed in Bishoftu town (NVI) from December 2020 to May 2021. Bishoftu exists at the south east of Addis Ababa with the distance of forty-seven kilometers. Bishoftu have an altitude of 9 ^{0} N and longitude of 40 ^{0} E at an altitude of 1850 meters above sea level in central high land of Ethiopia. The yearly rain fall of the town is around 866 mm of which 84% is in the extended showery period. The dry period lasts from October to February (CSA. 2018).

The societies are ever changing their living from agriculture since land has been employed by manufacturing. The town is one of growing industrial town of the country due to closeness to principal city named Addis Ababa and convenience for all types of infrastructures. NVI is the only company established in Bishoftu town for production of vaccines against animal disease in Ethiopia.

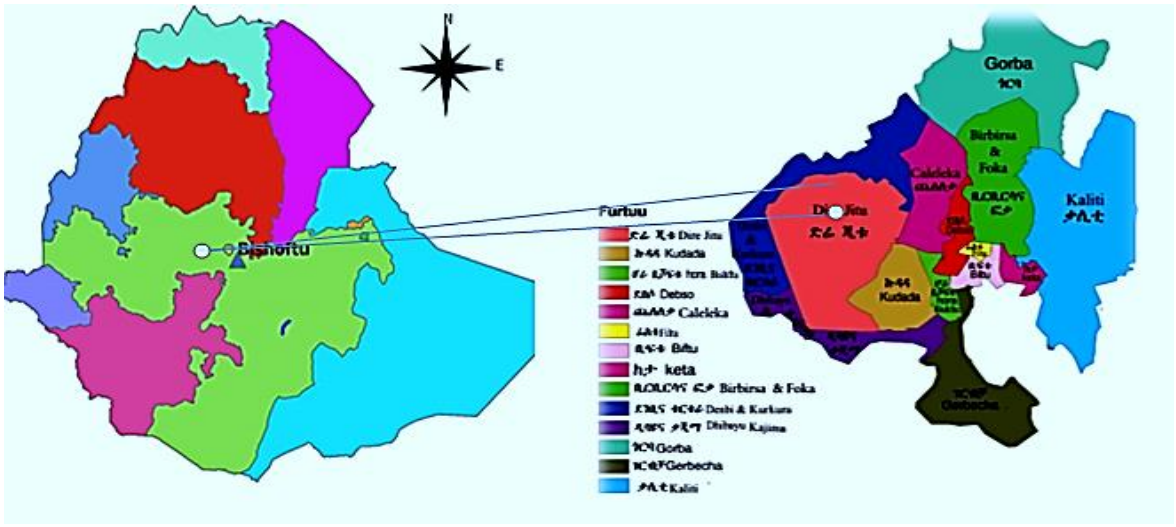


Figure 5: Study area created by Arch GIS

Source: (Arch map 10.2)

3.2. Research Methods

3.2.1. Study animal and design

Local breed of two infected cattle with black leg disease from the study area was used for the isolation of *Cl. Chauvoei.* Guinea pigs were used for preparation of freeze-dried Master Seed Bank from isolated strain in such a way that 115 of guinea pigs with three-hundred to four-hundred grams' weight. As of the total 115 guinea pigs; 100 of them were used for LD50 determination, 15 of them for challenging in which 5 of them were used as a control (Crichton *et al.,* 2006). At the same time for quality control or safety test of the new developed lyophilized master seed bank 3 cattle and 3 sheep with the age of one year for each animal from both species were used. Animals were reserved for one week to acclimatize to new milieu of sheds where the investigations are to be accomplished. The temperature of the animals was registered two times per day, and those viewing rise in body temperature were substituted (OIE, 2012).

3.2.2. Sampling method and technique

Purposive sampling method was implemented to gather sample from the two diseased cattle from Bishoftu specifically Dire Jitu kebele, and individual animals were selected relaying on prominent clinical signs of black leg like lameness, crepitant sound at hind limb and edema. Five types of samples were collected namely; edematous fluids, muscle tissue, spleen, liver tissue and heart blood. Therefore, 10 total samples were collected from the two diseased animals. Immediately after death *Cl. Chauvoei* was isolated in fresh black leg inoculum, blood agar and VF medium from heart blood, muscle, oedematous fluids. To collect the sample, the site of inflamed tissue with remedied spirit, at the center of the lesion was incised by means of sterile scalpel blade, and tiny a part of skin was taken out. The exposed part of the tissue was scorched with a warm spatula, then the aspiration fluid oedema by means of a pasture pipette was accomplished (Chakraborti, 2012). The heart of the cadaver was also sampled to collect blood under laminar air flow. Finally, the samples were transported to NVI under cold chain system for isolation and identification purpose.

3.3. Isolation of the organism

3.3.1. Cultural Method of Isolation and Identification

Two types of cultural medium were used, namely VF and liver Extract-Blood agar (Oxford, England). The whole procedure of bacteriological examination was done under a sterile state in a laminar flow to avoid contamination from the area. The composed samples specifically, blood sample was initially inoculated on to VF medium and incubated at 37 ^{0}C for forty-eight hours under anaerobic state using anaerobic jar. Test tubes consisting grown bacteria were taken and streaked on liver Extract-Blood agar (Oxford, England) in sterilized Petridish and incubated as completed earlier. Those colonies with identical morphology to *Cl. Chauvoei* were taken and subcultured on liver Extract-Blood agar in order to get pure organism (Williams *et al.,* 2014). Similarly, identification of the bacteria was done using three methods. These include identification through cellular morphology, biochemical test and cultural method. The cellular morphology by grams' stain was used to discover *Cl. Chauvoei* from *Cl. Speticum. Cl. Chauvoei* seen as citron, pleomorphic, with an individual chain, whereas *Cl. Speticum* reveals extended threads. Regarding biochemical test *Cl. Chauvoei* ferment sucrose but not salicin, whereas *Cl. Speticum* ferments salicin, yet not sucrose. Likewise, the cultural method was used in which *Cl. Chauvoei* is fastidious organism which necessitate glucose supplement than that of *Cl. Speticum* (Walker and Batty, 2013).

3.3.2. Biological method of isolation

In this case muscular tissues grinded in mortal was become ready at ten percent suspension in biological saline, then inoculated in to thigh muscle of guinea pigs with one ml dosage by intramuscular route. The animals have been patterned for prominent clinical signs such as dullness, depression and edematous swelling at injection site. The death of the animal was happened in twenty-four hours of inoculation. Immediately after death of the animal, the organism was isolated from heart blood under laminar flow (Cameron *et., al* 2010).

3.4. Molecular characterization of the organism

The pure growth of the bacteria on inoculum media was processed for molecular identification through PCR using specific primers flagellin gene of *Cl. Chauvoei* following Sasaki (2000) (Annex 2). DNA extraction was done using QIAGEN mini columns (QIAGEN, 2007). It is a costly but, an active technique of extracting extraordinary quality amplifiable genome from blood, urine dried blood spot, and buffy coat and tissue biopsy models. Samples under + 4 ^{o}c are taken to room temperature earlier starting to procedures (Annex: 1).

3.5. Preservation of the stain

Eventhogh there are two types of freeze-drying preservation method namely vegetative and spore form, in this specific study the spore form of preservation was considered for sustainable immunogenicity maintenance. Preparation, glyceration and freeze drying of spore were done according to OIE manual for manufacture of black leg vaccine (OIE, 2012).

3.5.1. Preparation of spore

Two times passage of identified *Cl. Chauvoei* strain was made on to VF medium with 12 hours' intervals and incubated at 37 ^{o}c under anaerobic condition. Eight hours post to the 2nd passage grams' stains was performed to confirm the purity of the passage, and only pure growth was inoculated on black leg inoculums in Erlenmeyer flasks, then incubated at 37 ^{o} c for 48 hours. The growth of the inoculums was checked by turbidity, tangent foam formation and confirmation of purity was checked by grams' stain (Henry, R. and Stampfli, 2014). The facilitation of sporulation was realized through maintaining the pure cultures at room temperature for twenty-one days. On twenty-one days of keeping, the cultures were harvested, that is by means of solitary layer of gauze the spore suspension was sieved. After checking the purity of the spore suspension using gram's stain, the pure suspension was agitated at five-thousand rotation per minute for one hours, and washed for

three times using sterile saline water. At the end, the dumped spore was combined with two times volume of physiological saline that is with the ratio of two to one of spore to saline water in to Erlenmeyer flask (OIE, 2012).

3.5.2. Glyceration of Spore

Glyceration of the spore was done to maintain the anaerobic environment of the prepared product. The formulation of glyceration of the spore was done in such a way that two times of ready-made spore or a mixture of spore and saline water was combined with sterile neutral glycerol (paraffin oil). Then, the combined product was maintained at plus four degree Celsius until the date of lyophilization (OIE, 2012).

3.5.3. Freeze drying of spore

The skimmed milk (HiMedia, India) becomes organized with five percent ratio to distilled water and autoclaved at 121 ^{o}c for 15 minutes. The same quantity of the sterile skimmed milk became combined with glycarated spore suspension of *Cl. Chauvoei* and freeze dried in one milliliter in vials. The lyophilization or drying process was done in which the primary drying in lyophilizer machine lasts about eighteen hours and the secondary drying accommodate four hours (OIE, 2012) (Annex 3).

3.6. Quality control tests of lyophilized strain

Lyophilized strain was directly checked for appreance of the cake in a vial, vacuum, moisture content and identity. However, for potency, sterility and safety test it is mandatory first to produce a vaccine batch from lyophilized strain.

3.6.1. Appreance and Vacuum test of lyophilized strain

The integrity of the vacuum was checked according to the NVI vacuum test procedure. First, checkup of weather the rubber stoppers was held firmly into the vial, and the top of the stopper should show a depression if it has been sealed under vacuum. Then the color of

all 16 vials freeze-dried pellet were checked if they do have the same color throughout. Similarly, the compactness and homogeneous after dilution was also determined. Finally, by using vacuum tester the vacuum teste was applied for all 16 freeze-dried pellets. All positive vials must display UV light in darkened room and all negative sample must display dark light. If the vacuum test is less than 75%, it requires further confirmation by residual moisture and potency test. Moreover, the freeze-dried product shall contain the appreance of a yellowish white dry cake easily mobile inside the container with homogenized and single cake (Martha *et al.,* 2016).

Interpretation of the result- the test is valid when 75% of the sample passes the vacuum test. For the physical appreance the entire test sample should fulfill the stated requirements (Martha *et al.,* 2016).

3.6.2. Moister content test of lyophilized strain

Five vials of freeze-dried vaccine seed bank were sampled to determine the moisture content material. The vials have been opened and the cake containing feasible organisms in the vials become disbursed on moister analyzer device containing phosphorous pentoxide. Then drying was completed at vacuum (0.01–0.03 mm Hg) at 56°C till a constant weight is attained. The acceptable moisture content of freeze-dried vaccine or seed bank should be below 3 % (Martha *et al.,* 2016).

3.6.3. Bacterial contamination test of lyophilized strain

The contamination test of lyophilized strain was checked by two test methods, namely morphological and cultural test methods. In the case of cellular morphological method three vials of freezed dried product were sampled and each vial was reconstituted using 1ml of sterile saline water. Pulling was done and the smear was arranged, stained by gram's stain and detected using microscope. It is expected that the seed-bank must not encompass any other bacteria apart from *Cl. Chauvoei* (Martha *et al.,* 2016). Similarly, in the case of cultural test method three different media and techniques were used, namely blood agar plates in aerobic environment, blood agar plates in anaerobic environment and

Thioglycolate fluid. 5 vials of the lyophilized product were streaked on 5 blood agar plates and incubated at 37 ^{o}c in anaerobic environment. The morphologies of the colonies were examined by using necked eye and a magnifying glass. At the same time the smear was prepared by gram's stain to examine under microscope. It is expected that the seed-bank must not encompass any additional bacteria apart from *Cl. Chauvoei.* Testing the seed-bank on blood agar slants and incubating under aerobic conditions at 37^{o}C for seven days is also essential. Then, the slant was examined for any growing. As *Cl. Chauvoei* is a strict anaerobe, there must not be any growth on blood agar slants. Thioglycolate fluid was also used in such a way that the sample was inoculated and incubated at 37^{o}C for 7 days. Testing the purity by smear examination daily up to seven days with Gram's method was applied. The seed-bank must not contain any other bacteria except *Cl. Chauvoei* (Martha *et al.,* 2016).

3.6.4. Identity test of lyophilized product

Similar PCR procedure used for identification by molecular characterization of isolated strain was implemented for identify test of lyophilized strain**.** One vial of lyophilized strain was reconstituted using 1 ml of PCR water and processed for molecular identification through PCR using specific primers flagellin gene of *Cl. Chauvoei* with 798 base pair following QIAGEN (2007) (Table 2). DNA extraction was done using QIAGEN mini columns. It is a costly but, an active technique of extracting extraordinary quality amplifiable genome from blood, urine dried blood spot, and buffy coat and tissue biopsy models. Samples under + 4 ^{o}c are taken to room temperature earlier starting to procedures (Annex: 1).

3.6.5. Determination of LD 50 of freeze-dried spore form

The LD 50 of the strain can be quality determined in sheep or livestock, but because of economic impact it is better to carry out in guinea pigs. Tenfold dilution with one dilution factor became prepared in a physiological saline solution comprising two percentage of CaCl2.2H2O. One milliliter of prepared product was inoculated in guinea pigs weighing three hundred to four hundred grams in such a way that from each tenfold dilution 10

gunia pigs were used so that total animals injected will be 100. Strict follow up was made for five days to record the number of animals dead from each dilution. Commonly for calculation of LD 50 two techniques are applied namely, Reed and Muench (1938) and Spearman-Karber (1908). However, recently there is an easy method to compute the 50% endpoint titer which is proposed by Muthannan (2016). Thus, in this specific study the latest or Muthannan method was used and the formula is as follows: Log_{10} 50% end point dilution = - [(total number of mortality/number of animals injected per dilution) + 0.5] × log dilution factor (Muthannan, 2016).

3.6.6. Sterility, safety and potency test of lyophilized product

Production vaccine batch is necessary to do other quality control tests like potency, sterility and safety, thus primarily batch of black leg vaccine was prepared from the lyophilized seed as per black leg vaccine production standard operative procedure (SOP) available at NVI.

Production of batch vaccine-Vacinal seed bank first passage was done on to 2 test tubes of VF medium and incubated at 37 ^{O}C for 12 hours. On the next day the purity and growth was checked by gram's stain and second passage was done on to 2 test tubes of VF medium and incubated at 37 ^{O}C for 6 hours to get more new young generations. Six hours later to the second passage the purity and growth was confirmed and it was inoculated on to 2 x 10 litters of black leg inoculums media then, incubated at 37 ^{O}C for overnight. On the next day the visual growth of the inoculated inoculum media was checked by turbidity and tangent foam formation. As usual the purity of the inoculum was confirmed by gram's stain (OIE, 2012).

Inoculum pure culture passage was done on to 10 x 10 litters of black leg production media prepared by filtration using EK2 filter pad. Then it was incubated at 37 ^{O}C for 24 hours. Similar to the of inoculum media the growth and purity was checked by turbidity, tangent foam formation and gram's stain. Inactivation of the production culture was done using 0.7 % of formaldehyde and all bottles were agitated manually for 6 days continually. Finally, inactivation test, adjuvating with aluminum potassium Sulphate, safety and sterility tests of final product was done following available procedures (OIE, 2012).

Sterility test of the batch vaccine

The sterility tests of the inactivated black leg vaccine produced from lyophilized product was done using medium like Tryptose agar, thioglycolate USP fluid, soubroad agar and tryptic soya broth (SBCDM) (Table **7**). The incubation time of all tests is 7 days except soubroad agar which requires 14 days (Aberu and Uzel, 2016).

Safety test of the batch vaccine

The safety test of the formaldehyde inactivated vaccine should be carried out either on sheep or cattle. Three non-vaccinated against black leg sheep with one year of age were used. Each animal was injected with 5 ml of vaccine produced from the seed by using subcutaneous rout at inner face of the thigh: follow up was made for 10 consecutive days. Similarly, cattle were used in such a way that three non-vaccinated against black leg with one year of age. 10 ml vaccine prepared from the seed bank was given in the neck by subcutaneous route and observation was made for ten consecutive days. In both species the seed-bank fulfill the test if there is no unfortunate response excluding minor inflammation at the site of vaccination that diminishes in four to five days (Martha *et al.*, 2016); (Frey and ferlquent, 2010).

Potency test of the vaccine batch

Challenging dose-Produced vaccine using lyophilized seed bank was thoroughly homogenized by shaking in a vial. Following Crichton *et al.*, (2006) 10 gunia pigs were vaccinated with 1ml dose subcutaneously in ventral thoracic area on the right side. Booster dose was given after 21 days of the first vaccination with the same dose and route of administration but on the right side following the initial vaccination. Finally, 14 days

later to the booster dose all vaccinated and 5 unvaccinated control were challenged with challenge strain available at NVI in such a way that each were injected intramuscularly at ventral thoracic area with determined LD-5O dose spores of virulent challenging strain activated with calcium chloride.

4. RESULTS

4.1. Isolation and Identification

4.1.1. Cultural Characteristics of Cl. Chauvoei

The cultural characteristics of *Cl. Chauvoei* are presented in (Table 7). The morphological futures of the cells of the bacterial species were found with single or paired long and short rods with rounded ends. Sporulation of cells was also observed by grams' stain when the studied long preserved culture was seen under microscope (Figure 7).

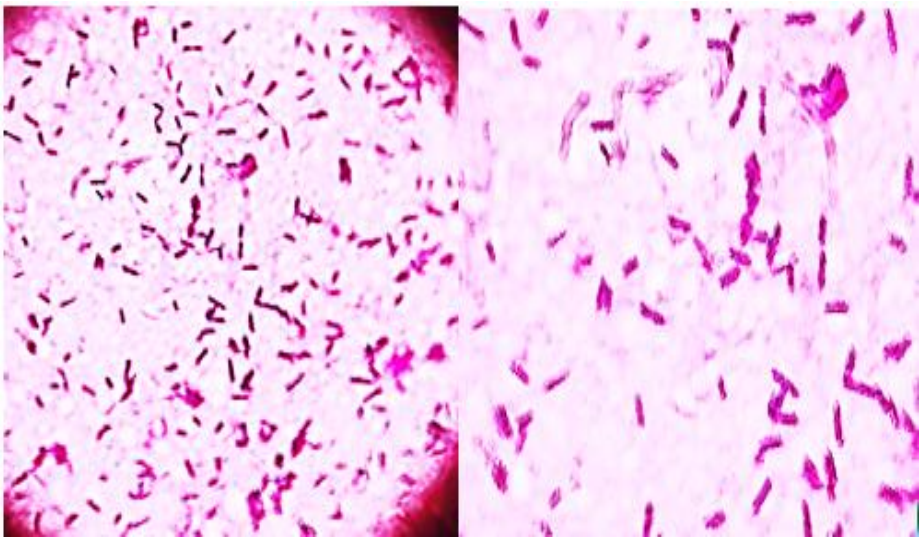


Figure 6: Gram's stain isolated *Cl. Chauvoei*

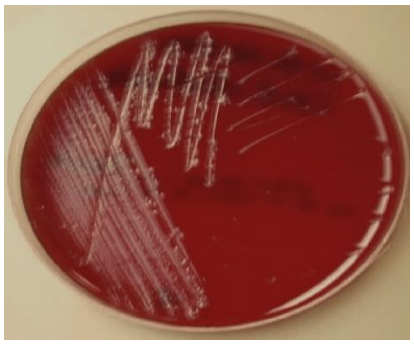


Figure 7: *Cl. Chauvoei* hemolytic colonies on blood agar

The bacteria formed large, whitish grey irregular colonies having a zone of hemolysis with whole margin colonies on blood agar medium (Figure 8).

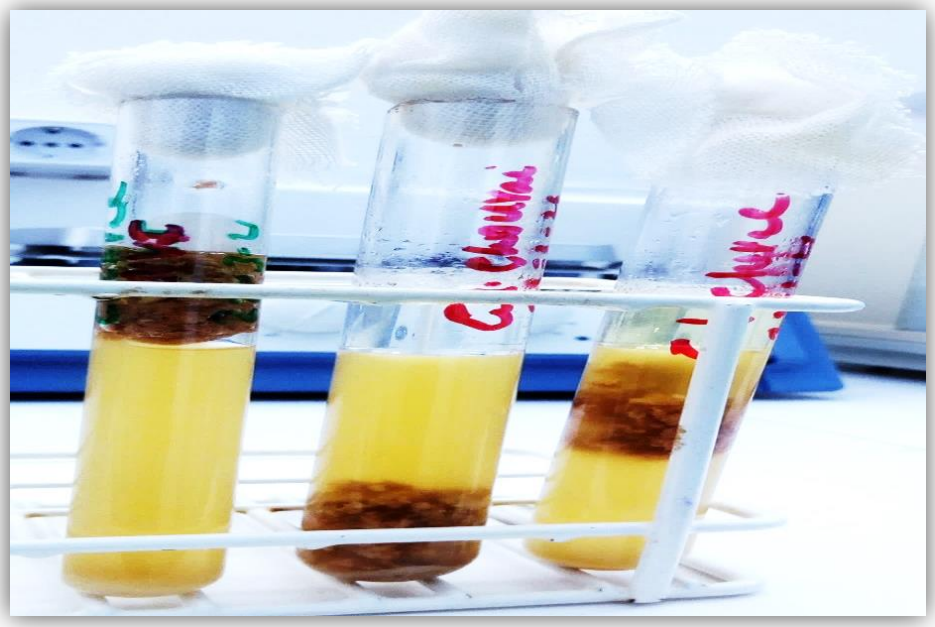


Figure 8: *Cl. Chauvoei* isolated on VF (viande-foie) media

Similarly, on VF medium the organism growth cause turbidity of culture and tangent foam formation with meat extract reverting from the bottom of the broth to the top with ring formation under paraffin oil (Figure. 9).

Table 1: The cultural morphological and staining characteristics of *Cl. Chauvoei*

Shape	*Arrangement*	*Gram stain*	*Colony future*	*Cultural future*	*Spore staining*
long/short rods	Single or pair or pleomorphic	Gram + ve but appears as Gram-ve in old culture	Whitish-gray with hemolysis	Reversion, turbidity, foam formation	Distended at the site of spores

4.1.2. Biological properties of Cl. Chauvoei

With the purpose of additional confirmation like pathogenicity study and inocucity test, the isolates were injected to a few guinea pigs. All the samples brought about hemorrhages within the heart and mortality of the animals after 24 hours. The cultural morphology and appreance result were also comparable with that of the true black leg. Replication of all of those tests on samples of died guinea pigs showed the isolates have been clostridial (Figure 10)

Figure 9: Re-isolation of *Cl. Chauvoei* from Guinea pigs

The figures show that from left to right; the left one is while re-isolating from guinea pig, and inoculation of the organism directly in to black leg inoculum under sterile air using laminar flow at NVI. The middle one was used as a control without inoculation, while the right one is the inoculated one with bacteria from gunia pigs, and after 24 hours of incubation at 37 ^{0}C (There is a visible turbidity and tangent foam formation).

4.1.3. Biochemical properties of Cl. Chauvoei.

The biochemical investigations were intended to register very specific nature of *Cl. Chauvoei* because a number of species of the same family are morphologically very comparable to distinguish them from one another. The biochemical tests like; glucose, maltose, lactose, sucrose, salicin, catalase and indole test results are summarized in (Table 9) (Figure 11).

Table 2: Biochemical result of the organism

Biochemical tests	*GU*	*MA*	*LA*	*SU*	*SA*	*CT*	*IN*
Result	+VE	+VE	+VE	+VE	-VE	-VE	-VE

Gu= glucose, MA= maltose, LA= lactose, SU=sucrose, SA=salicin CT=catalase, IN=indole, -VE= negative, +VE= positive.

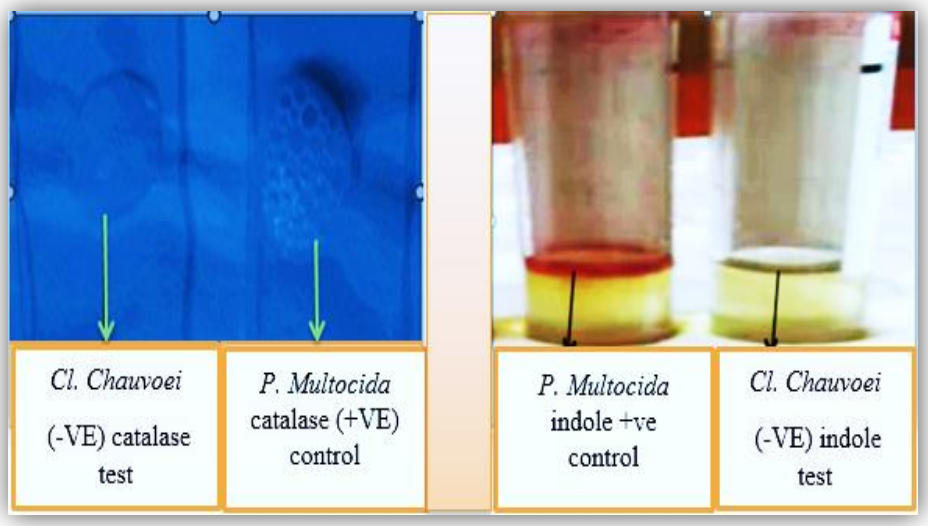


Figure 10: Catalase and indole tests of *Cl. Chauvoei*

Cl. Chauvoei is catalase negative thus there is no bubble formation in the test result, but the positive control with *P. Multocida* indicates it is catalase positive. Similarly, the indole positive control test with *P. Multocida* there is ring formation but, *Cl. Chauvoei* Indole test revealed no ring formation as it is indole negative.

4.2. Lyophilization of identified *Cl. Chauvoei*

With the aim of spore form preservation of the isolated strain, the Lyophilization or freeze drying of *Cl. Chauvoei* was done and all quality control tests like sterility, vacuum,

moisture content and determination of LD 50 for challenging and identity tests were achieved. Finally, labeling was performed.

Figure 11: The new developed and old preservation method of *Cl. Chouvae*

Letter A indicates the new developed technique spore form of *Cl. Chauvoei* preservation method which was achieved at NVI for the first time to improve quality of vaccine mainly immunogenicity. As one can see from the picture, the cake form of the product was done in such away, 5 ml volume capacity of vials were used in which each vial consists of 1 ml of spore form of culture, 0.5 ml of paraffin oil and 1.5 ml of milk stabilizer. Rubber stopper with stainless steel capsule was used during capsulation. Figure **B** indicate the old preservation method with test tubes consisting of 9 ml of the mixture of bovine liver-meat extract, Tryptose broth, liquid oil paraffin and 1 ml of spore form of culture. The product stopper was simply gauze, so it is very prone to even contamination.

4.3. Quality control tests results of lyophilized strain

4.3.1. Contamination test result of lyophilized strain

The morphological futures of the freeze-dried strain by gram's stain under microscope showed no other bacteria apart from *Cl. Chauvoei.* The lyophilized organism by grams' stain showed gram positive rods with rounded ends, and large cigar shaped rods which is

identical to the morphology of *Cl. Chauvoei.* Similarly, up on culturing the product on blood agar plates it showed identical morphology to that of *Cl. Chauvoei.* No growth was perceived on those incubated in aerobic circumstance. Likewise, no growth was detected on Thioglycolate medium.

4.3.2. Appreance, vacuum and moister content test result of lyophilized strain

From all 16 vials samples of freeze-dried pellet 15 of them displayed UV light in darkened room during detecting by using vacuum tester. So, the vacuum test was 93.75 % which is acceptable as it is greater than the minimum acceptable result (75%). At the same time, the moister content of the lyophilized product was 1.5 %, which is acceptable since it is less than 3%. Moreover, up on visual inspection, the freeze-dried product contains the appearance of a yellowish white dry cake easily mobile inside the container with homogenized and single cake.

4.3.3. Identity test result

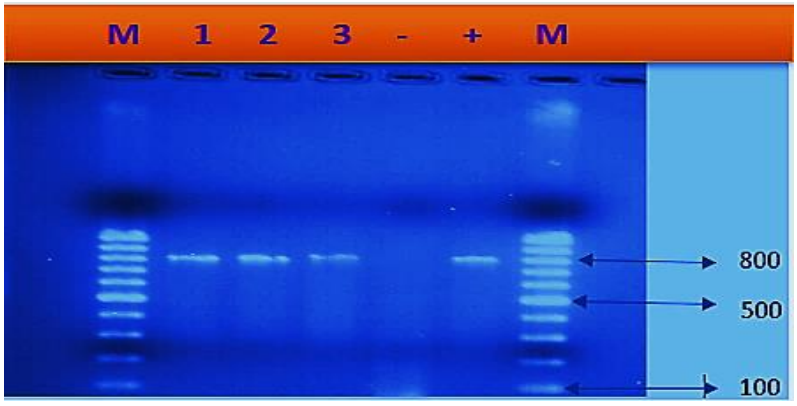


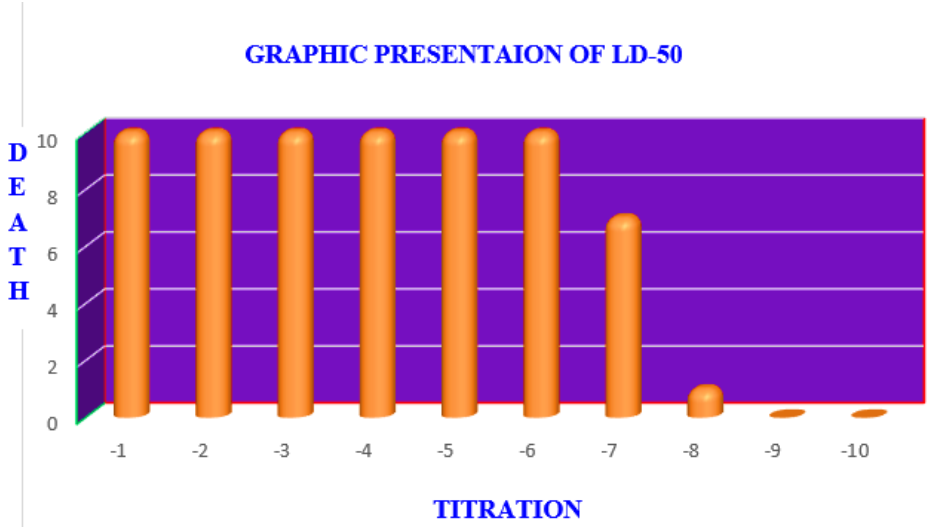
Figure 12: Conventional PCR using primers for *Cl. Chouvae* characterization

M represents molecular marker, lane with number (1-3) signifies extract from the *Cl. Chouvae* amplified using flagellin gene specific primers (forward primer (FlaF) and reverse primer (FLaCCR), - (ve) denotes negative control, and + (ve) symbolizes positive control.

4.3.4. Determination of LD 50 of freeze-dried spore form

Table 3: LD-50 determination of the freeze-dried spore of *Cl. Chouvae* on Guinea pigs

Log 10 bacterial dilution	*Guinea pigs*		
	Died	*Inoculated*	*Death ratio*
-1	10	10	10/10=1
-2	10	10	10/10=1
-3	10	10	10/10=1
-4	10	10	10/10=1
-5	10	10	10/10=1
-6	10	10	10/10=1
-7	7	10	7/10=0.7
-8	1	10	1/10=0.1
Total	*67*	*100*	6.7



Formulation to compute $\log_{10}$ 50% end point dilution**:** = - [(total number of mortality/number of animals injected per dilution) + 0.5] × log dilution factor.

= - (67/10 + 0.5) x 1 = -7.2; 50 % end point dilution = $10^{-7.2}$

The titer of the freeze-dried strain= $10^{7.2}$ LD50/ML.

4.3.5. Sterility test result of the vaccine batch

Table 4: The sterility test result of black leg vaccines batch

No	*Type of medium*	*unite*	*No. of tests*	*Dose inoculated*	*Result*		*Conclusion*
					+(ve)	*-(ve)*	
01	Tryptose agar (37 ^{O}C)	plate	2	1 ml each	0	2	*Pass*
02	SBCDM (37 ^{O}C)	plate	3	1 ml each	0	3	
03	Thioglycolate (37 ^{O}C)	ml	3	1 ml each	0	3	
04	soubroad agar (25 ^{O}C)	plate	1	1 ml	0	1	

4.3.6. Safety test result of vaccine batch

In both cattle and sheep, the vaccine batch produced from the lyophilized strain did not shown any adverse reaction except mild local swelling at injection site. Similarly, during 15 days of follow up on post vaccination the body temperature of the animals remains normal.

4.3.7. Potency test result of vaccine batch

Table 5: Showing potency test result and its interpretation after challenge dose.

Animal group	*No. challenged*	*Vaccination volume (dose/ml/)*	*Challenge dose (LD 50)*	*Observation post challenge*		*Conclusion*
				Dead	*Survived*	
Uncontrolled	10	1ml	$10^{6.8}$	1	9	90%protection
Controlled	5	-	$10^{6.8}$	5	0	No protection

5. DISCUSSION

Clostridial microbes are essential pathogenic organisms causing a multiple disease in several domestic animals. The most important is black leg resulting in huge economic loss. This study was performed at Bishoftu area specifically at NVI amid to develop new preservation method for *Cl. Chouvae* to improve the quality of black leg vaccine produced at NVI. *Cl. Chouvae* was isolated and identified from suspected cases of cattle in Adea Liban district by conventional microbiological method. *Cl. Chouvae* was cultured on several culture media and all cultural and associated features were documented (Table 8). Comparable features were detected in PYG broth medium by Singh (2001) and Quninn *et al.,* (2004).

The cell morphology by gram's staining reveled presence of gram-positive rods with rounded ends, and large cigar shaped rods or citron form which was also indicated to be by Useh *et al.,* (2006) and Jubb *et al.,* (2015). Moreover, the characteristics of the isolated organisms with Gram positive and morphologically rounded ends, as well as the selected microbes on Blood agar with grayish, colonies and with presence hemolysis zone that looks likes the uniqueness colonies of *Cl. Chouvae* as described by Gerhardt *et al.,* (1994). A number of biochemical tests were carried out to record the biochemical property of *Cl. Chouvae* (Table 9). Marchanet (2012) also established similar result regarding biochemical proprieties excluding catalase test to be negative. Some environmental influences can also play role to alter its biochemical activities. However, kanwar *et al.,* (2016) found exactly comparable result to the current biochemical properties discovery. The organisms were found positive for sucrose fermentation but negative for salicin fermentation. The results of these biochemical tests suggested that the isolated organisms could be considered as *Cl. Chouvae* rather than *Cl. Speticum.* Hogan and Bruner (2008); Langroudi *et al.,* (2012) were also reported similar biochemical characteristics.

With the aid of further confirmation like pathogenicity study and acuity estimation on guinea pigs the selected organisms showed death and hemorrhage in the heart up on

postmortem examination which is typical characteristics of pathogenic effect of *Cl. Chouvae* as described in Piers *et al.,* (2012). Intended for approval of isolated bacterium as the primary cause black leg sickness the PCR test was completed constructed on Sasaki (2000). PCR was run to validate the isolate as *Cl. Chouvae* by means of the specific primers (Table 2). PCR remains 100% precise and sensitive for isolate of *Cl. Chouvae* cultures with the predominant flagellin antigen identified by the amplification of a 798 bp fragment which is comparable to the result obtained by Sasaki (2001); Uzal *et al.,* (2003); Bagge *et al.,* (2009) and Abreu *et al.,* (2016).

The new preservation method of the isolated and identified *Cl. Chouvae* as a master vacinal seed bank was done based on OIE (2012) manual. It was arranged in skimmed milk and freeze-dried in one milliliter amount in vials by adding of the commended quantity of liquid glycerol or paraffin oil to preserve anaerobic situation of the organism. All the quality control test results of the lyophilized product such as vacuum test (97.5), moisture content (1.5%), the LD50/ML of freeze-dried spore strain ($1 \times 10^{7.2}$), potency (90%), has an appreance of yellowish white dry cake easily mobile with in a vial, sterile, safe, free of contaminant and identity pass. These all-test results fulfil the result mentioned on OIE (2012) manual which says the vacuum must be greater than 75 %, moisture content less than 3%, potency must be greater than 75 % protective and LD-50 should be greater than or equal to 1×10^{7}. According to wood (2018) with freeze-dried products boosting expiration date up to 20 years, it can extend our product life time, minimizing write-offs owing to expiry. Similarly, freeze-dried spore as a master seed bank for black leg vaccine production stored at -20^{0} C retain viability for years, however stability test at diverse storage temperatures for extended period of time is vital to accomplish this study. Furthermore, study reinforced by DNA sequencing is likewise indispensable to conclude these inferences.

6. CONCLUSION AND RECOMMENDATIONS

This is the principal study in Ethiopia, which considers about the developing new preservation method of *Cl. Chouvae* for the best immunogenic black leg vaccine production. It was concluded that the isolated, identified and lyophilized strain was potent and fulfilled the quality criteria mentioned on OIE (2012) manual. The isolates were also highly pathogenic to gunia pigs. This study constitutes a crucial and initial step allowing in designing or improving the effectiveness and efficiency of the existing vaccine to support black leg disease controls in the country. Therefore, based on above conclusions the following recommendations were forwarded:

- → Stability test at diverse storage temperatures for extended period of time is vital to confirm the persistent retention of viability of lyophilized product.
- → The molecular analysis should be reinforced by the like DNA sequencing as to confirm *Cl. Chouvae* strains that will ultimately helpful in scheming an effective and efficient vaccine.
- → Post vaccination immunogenicity test by serological method is crucial to evaluate the protective capacity of vaccine from lyophilized product.

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ANNEXES

ANNEX 1: **DNA EXTRACTION USING QIAGEN $_{MINI}$ COLUMNS**

Extraction protocol:

- Complement 20µl of QIAGEN Protease in to microcentrifuge tube with 1.5 milliliter volume size.
- Complement 200µl sample to the tube.
- Complement 200µl buffer AL to the sample in microcentrifuge.
- Incubate in water bath at fifty-six ^{0}C for 10 minutes.
    - DNA yield extents a maximum after layis for ten minutes at fifty-six ^{0}C, however elongated incubation periods will not have undesirable effect on DNA extraction.
- Complement 200µl of ethanol and homogenizing by means of pluse-vortexing.
- Handover the combination in to QIAamp spin column and agitate at 8000 rpm for one minute.
- Wash the mix two times using 500 µl buffers. In the first wash stir the mix and buffer collected at 8000 rpm for one minute. Place the QIAamp spin column in a clean 2 ml collection tube, and remove the collection tube holding the filtrate. In the 2nd wash reprise the similar procedure, but stir at 14000 rpm for three minutes.
- Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube and abandon the collection tube comprising the filtrate. Add 50 µl buffer AE or distilled water. Incubate at room temperature for 5 minutes and then filter at 8000 rpm for one minute.
    - Another elution stage with additional 50 µl buffer AE will upsurge harvests by up to 15%.

ANNEX 2: **CONVENTIONAL PCR *CL. CHOUVAE* IDENTITY TEST**

Master mix preparation

S. N	Type of reagent	For one reaction	Total reaction	Re
1	RNase free water	2 µl	12	
2	Primer-Clchfla-AGAATAAACAGAAGCTGGAGATGA-FOW	2 µl	12	
3	Primer-Clchfla-TACTAGCAGCATCAAATGTACC-REV	2 µl	12	
4	IQ Supermax	10 µl	60	
5	Add template	4 µl		
	Total volume	20µl		

Run PCR reaction

Steps	Temperature	Time	Cycle	Rem.
Initial denaturation	95 ^{0}C	5 minutes	1 cycle	
Denaturation	95 ^{0}C	45 second	35 cycle	
Annealing	55 ^{0}C	1 minute		
Elongation	72 ^{0}C	1 minute		
Final elongation	72 ^{0}C	7 minutes	1 cycle	
Put at	4 ^{0}C	Until machine off		

A. Agarose gel preparation

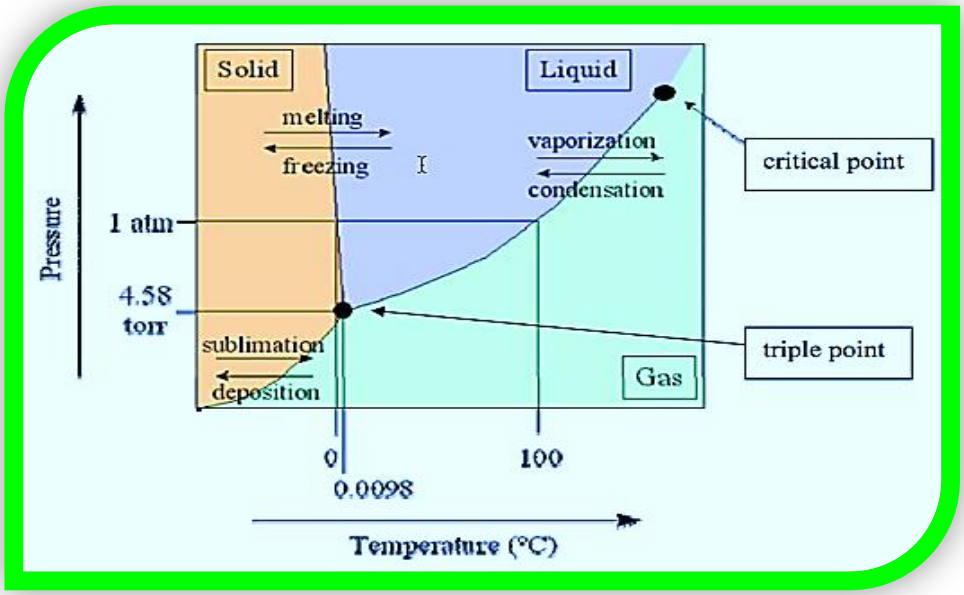
SN	**Activates**	**Remark**
1	Make ready of 2% Agarose gel	
2	Complement 5 µl gel red with 4 µl dye and 10 µl ladder	
3	Run electrophoresis for 1:20 hours at 120 V	
4	Read the result using UV light	

ANNEX 3: **VACCINE LYOPHILIZATION PROTOCOL**

- Formulation of spore, milk stabilizer and paraffin
- Dispensing the product using Vials
- The product will be loaded
- Thermal Treatment: at atmospheric pressure the product will be frozen.
- Sublimation: primary drying using vacuum
- Desorption: secondary drying using vacuum
- Backfill and stoppering of product in vials under partial vacuum
- Taking away of dried product from freeze dryer

Sublimation in the freeze-drying method can be defined merely as:

- FREEZE - The product in vials is totally frozen.
- VACUUM - The product frozen is then placed under a deep vacuum.
- DRY – Heat energy is then supplemented to the product instigating the ice to sublime.



Freez drying/lyophilization information: Basic principle

ANNEX 4**: STERILITY TESTING MEDIA**

TRYPTIC SOY BROTH

Formulation:

The tryptic soy broth powder was prepared with the following ingredients in g/L

Casein peptone	17.0
Soya peptone	3.0
NaCl	5.0
Dipotassium phosphate	2.5
Dextrose	2.5

Preparation procedure

- Weight 30g of the tryptic soy broth powder
- Add the measured powder in 1000ml of distilled water
- Dissolve well using magnetic stirrer
- Distribute 9ml of the solution into sterile test tubes
- Sterilize by autoclaving at 121 ^{0}C for 15 minutes
- Incubate at 37 ^{0}C for 24 hours to check its sterility
- Store at room temperature

SCDM

Component:

Casein peptone	17gram/litter
Soya peptone	3gram/litter
NaCl	5gram/litter
Dipotassium hydrogen phosphate	2.5 gram/litter

Preparation procedure:

- Add 30 gram of dehydrated medium in one-liter distilled water
- using regular agitation and heat for 1 minute
- Autoclave at 121^0C for 15minutes
- Calm at 45 – 50^0C
- Shake to homogenize and dispense using sterilized test tubes.

TRYPTOSE AGAR PREPARTION

Formulation:

Glucose	1 gram
Agar	22 grams
Tryptose	20 grams
Sodium chloride	5 grams

Formulation steps:

- Measure the required amount of each component used in preparation of Tryptose agar.
- Mix with required amount add distilled water.
- Agitate using magnetic stirrer for about 30 minutes.
- Homoginize using heat.
- Using last containers dispense it.
- Using autoclave steralize it at121^0C for 15 minutes
- Label it.
- Store under +4^oC.

SABOROUD AGAR FORMULATION

Preparation:

Agar	15 gm
Glucose	40 gm
Peptone	10 gm

The ready-made saboroud agar medium formulation is as follows :

Powder form of Saboroud agar	30 grams
Distilled water	1000 grams

Formulation steps:

- Measure the required amount of each component used in preparation of Saboroud agar Mix with required amount add distilled water.
- Agitate using magnetic stirrer for about 30 minutes.
- Homoginize using heat.
- Using last containers dispense it.
- Using autoclave steralize it at121^0C for 15 minutes
- Label it.
- Store under $+4^o$C.

ANNEX 5 : **TEMPERATURE RECORD**

Temperature record for saftey test of balck leg vaccine batch /cattle/

	Control group				Saftey tested (vaccinated)					
Day	B-05		B-25		B-40		B-41		B-42	
	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.
1	38.2	39.1	38.4	39.0	38.0	39.0	38.4	39.0	37.7	39.2
2	37.9	39.1	37.2	39.0	37.8	39.3	38.2	39.1	37.8	39.1
3	37.8	39.3	37.5	39.2	37.7	39.4	38.1	39.2	38.2	39.2
4	37.7	39.0	38.4	39.3	37.9	39.0	37.9	39.0	37.8	39.1
5	37.8	39.0	38.2	39.0	38.0	39.0	38.0	39.3	37.6	38.1
6	37.5	38.9	37.9	39.0	38.2	39.2	38.0	40.1	37.9	39.0
7	38.0	39.1	37.8	39.1	38.4	39.3	39.4	39.0	37.8	39.1
8	38.0	39.0	37.3	40.0	37.9	38.9	38.1	39.0	38.5	39.2
9	38.6	39.0	38.1	39.0	37.9	39.0	37.8	39.2	37.9	39.3
11	37.8	39.2	38.4	39.3	38.1	39.0	37.9	39.3	37.8	39.2
12	38.3	39.3	38.0	39.0	37.8	39.0	37.6	39.2	37.9	39.0
13	38.1	39.0	38.5	38.9	38.0	39.2	38.8	39.2	38.8	39.2
14	37.7	39.1	38.0	38.8	38.0	39.3	39.3	39.2	37.7	39.1

Temperature record for saftey test of balck leg vaccine batch /Sheep/

	Control group				Saftey tested (vaccinated)					
Day	B-35		B-36		B-37		B-38		B-39	
	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.	Mor.	Aft.

1	38.2	39.3	38.4	39.0	38.0	39.4	38.4	39.0	38.2	39.4
2	38.9	39.4	37.9	39.0	38.3	39.3	38.2	39.4	37.9	39.1
3	38.8	39.3	38.5	39.2	38.4	39.0	38.1	39.0	38.2	39.2
4	37.4	39.0	38.4	39.3	38.5	39.0	38.3	39.0	38.4	39.1
5	38.2	39.0	38.2	39.0	38.0	39.0	38.0	39.3	38.6	39.2
6	38.5	38.9	37.9	39.0	38.2	39.4	38.0	39.2	38.9	39.3
7	38.0	39.1	38.8	39.1	38.4	39.3	38.4	39.3	38.4	39.1
8	38.0	39.0	38.3	40.0	38.4	38.9	38.1	39.9	38.5	39.5
9	38.6	39.0	38.1	39.4	37.9	39.0	37.8	39.2	38.6	39.4
11	38.8	39.2	38.4	39.3	38.1	39.0	37.9	39.3	38.8	39.5
12	38.3	39.3	38.0	39.0	38.4	39.0	38.6	39.4	37.9	39.9
13	38.1	39.0	38.5	39.0	38.0	39.2	38.8	39.9	38.8	40.0
14	37.9	39.1	38.0	39.0	38.0	39.3	39.3	40.0	37.9	39.1