National Bovine Brucellosis Prevention, Control and Elimination Plan - 2022

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Recommended citation

NCAH. (2022). *National Bovine Brucellosis Prevention, Control and Elimination Plan.* National Centre for Animal Health (NCAH), Department of Livestock, Ministry of Agriculture and Forests, Royal Government of Bhutan.

Acknowledgements

The following officials under the Department of Livestock are acknowledged for their contributions in developing this plan document

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MoAF/Sec/07/2021-2022/ *548* June 17, 2022

Foreword

Brucellosis is a multi-species zoonotic disease which affects a wide range of animal species. It causes abortion in cattle, birth of weak or dead calves and infertility resulting in economic losses associated with reduced productivity and trade impediment. In addition, brucellosis is a highly infectious zoonotic disease which causes undulant fever or Malta fever in humans. Brucellosis, therefore, is a disease of significant economic and public health concern.

Although the current prevalence of disease is low (<5%), with the intensification of dairy farms and movement of animals at increased frequency associated with dairy development in the country, there is risk of widespread infection among the cattle population. We have learnt through practical experience that responding to animal disease outbreaks is an exhaustive and resource intensive process. Therefore, it is necessary that we deal with livestock diseases in a systematic and strategic manner to minimize the socio-economic and public health impact resulting from outbreaks and ultimately achieve elimination. In doing so, it is necessary that there is response plan document outlining systematic approaches in responding to outbreaks, identifying relevant stakeholders and their roles and responsibilities, fund mobilization mechanism and monitoring and evaluation of the control plans.

In this regard, I would like to thank the National Centre for Animal Health and the Animal Health Division, Department of Livestock, for taking the lead in developing the "National Bovine Brucellosis Prevention, Control and Elimination Plan-2022". I also extend my appreciation to all individuals who have contributed towards developing this plan document. I am sure this document will serve as ready reference to all the stakeholders involved in the prevention, control and elimination of brucellosis in the country. Further, I am confident that this document will directly contribute to minimizing the incidence of bovine brucellosis and achieving elimination of brucellosis from the cattle population in the country.

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1 Background

Brucellosis is a multi-species zoonotic disease which affects a wide range of animal species including humans. It causes abortion in cattle, birth of weak or dead calves and infertility resulting in economic losses associated with reduced productivity and trade impediment. In Asia and Africa, the reported prevalence of brucellosis in cattle ranged from 0 – 68.8% and among the high-risk human populations, such as veterinarians, livestock handlers, and abattoir workers, the average prevalence was 11% suggesting that brucella species is a major cause of disease in humans and animals. In Bhutan, currently, there are only sporadic cases of *Brucella spp.* infection in cattle with low prevalence in the country. Through a concerted effort, the disease can be eliminated from the animal population of Bhutan. Nevertheless, it can become a serious problem for both animals and humans in future if appropriate interventions are not made in time. Therefore, it is crucial to invest resources at this stage to combat the disease. This can be done through implementation of **National Bovine Brucellosis Prevention, Control and Elimination Plan 2022.** The implementation of this elimination program requires committed fund support and resources. There is also a need for a strong policy support to acquire funds for compensation of culled animals.

This plan is applicable for the prevention of brucellosis incursion, preparedness and control in the event of brucellosis outbreak and gradual elimination of the disease from the country. This plan can also be adopted to respond to outbreaks of brucellosis in small ruminants and pigs. The document is prepared after the rise in incidence of brucellosis in dairy cattle and few reported cases of human infection in the country. This document is developed to ensure that all the required resources, expertise, and services are efficiently and rapidly mobilized during suspected or confirmed outbreak of brucellosis to keep the morbidity, mortality, and social disruption resulting from the outbreak to the minimum.

1.1 Objectives

- To strengthen surveillance for early warning, detection and response to brucellosis outbreak
- To rapidly contain or prevent spread of the outbreak at the source;
- To reduce the risk of human infection;
- To enhance trade of animal and animal product
- To achieve elimination of the disease from the country

2 Nature of the disease

2.1 History of the disease

The organism (bacteria) causing the disease was first identified by Dr. David Bruce in 1887 during the Crimean war. And in 1897, Dr. Bernhard Bang identified *Brucella abortus*. Therefore this disease is named as brucellosis as well as Bang's disease.

2.2 Brucellosis as an infectious disease

It is a contagious disease of livestock with significant economic and public health impact. It is caused by various bacteria of the genus *Brucella*. It affects cattle, swine, sheep, goats, camels, dogs, other mammals including marine mammals and humans. The disease in animals is characterized by abortions or reproductive failure. While animals typically recover, and will be able to deliver live offspring following the initial abortion, they may continue to shed the bacteria. Brucellosis in cattle (*B. abortus*) in sheep and goats (*B. melitensis*) and in swine (*B. suis*) are diseases listed in the World Organisation for Animal Health (WOAH) Terrestrial Animal Health Code and must be reported to the WOAH (Terrestrial Animal Health Code).

2.3 Transmission and spread

Species	Main Animal Host(s)
B. abortus	Cattle
B. melitensis	Goats, sheep, camels
B. suis	Pigs
B. canis	Dogs
B. ovis	Sheep, goats
B. neotomae	Woodrats
B. pinnipediae	Pinnipeds (seals, sea lions, walruses)
B. ceti	Cetaceans (dolphins, porpoises, whales)
B. microti	Common vole

Brucellosis is typically spread when the animal aborts or gives birth. High levels of bacteria are found in the birth fluids of an infected animal. The bacteria can survive outside the animal in the environment for several months, particularly in cool moist conditions. They remain infectious to other animals which become infected by ingesting the bacteria. The bacteria also colonise the udder and contaminate the milk. The disease can also infect animals and humans through cuts in the skin, or through mucous membranes. Brucellosis is an important disease in wildlife, infecting

feral pigs, bison, elk and European hares. The reservoir of disease in wildlife complicates eradication efforts. The bacteria have also been found in marine mammals.

2.4 Public health risk

Brucellosis highly infectious for humans causing a disease called undulant fever or Malta fever, since it was first recognised in Malta during the 1850s. Symptoms in humans include intermittent or irregular fever, headache, weakness, profuse sweating, chills, weight loss and general aching. Infections of organs including the liver and spleen may also occur. Veterinarians, farmers, and abattoir workers are vulnerable to infection as they handle infected animals and aborted foetuses or placentae. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as abortion materials. The disease can also spread to people through consumption of unpasteurised milk coming from infected animals.

2.5 Clinical signs

Typically the disease is mild, with the infected animal showing few signs until abortion. There may be swelling of the testicles in males, and occasionally the bacteria localizes in the joints causing arthritis. In horses, it causes a condition called fistulous withers or poll evil, a swelling of the neck or back. However pregnant mares may either abort or give birth to weak and vulnerable foals. The importance of Brucellosis is that it causes poor reproductive performance, due to abortions, infertility, retention of placenta, stillbirth or birth of weak offsprings. It results in huge economic losses to dairy, sheep, goat and pig farmers.

2.6 Diagnosis

All cases of abortion as well as orchitis in cattle should be considered as suspected brucellosis and should be investigated through the herd history and submission of specimens for laboratory testing. The clinical signs of brucellosis are not pathognomonic and confirmation of *Brucella* infections can be made only by the isolation and identification of the organism. However, when bacteriological examination is not practicable, diagnosis must be based on molecular or immunological tests. The test used for diagnosis of brucellosis is provided in the **table below**

Method	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of suspect or clinical cases	Herd/flock prevalence of infection – surveillance
Agent identification					
Staining methods	-	-	-	-	-
Culture	-	-	-	+++	-
PCR (Polymerase chain reaction)	-	-	-	+/++	-
Detection of immune response					
Buffered *Brucella* antigen test (Rose Bengal Test or Brucella plate agglutination test)	+++	++	+++	+	+++
Fluorescence Polarization Assay	++	++	+	++	++
Complement Fixation Test	++	++	+++	++	+++
Indirect-ELISA	+++	++	+++	++	+++
Competitive-ELISA	++	+	+	+	++
BST (Brucellin skin test)	++	–	+	+++	++
SAT (Serum agglutination test)	++	+	+	–	+
NH (Native hapten) and cytosol protein-based tests	-	-	+	++	-
Bulk milk tests Milk I-ELISA or Milk ring-test	+++	-	+++	+	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability,
or other factors severely limits its application; – = not appropriate for this purpose.

2.7 Prevention and control

Surveillance using serological tests, as well as tests on milk e.g., the milk ring test, can be used for screening and play an important role in campaigns to eliminate the disease. As well, individual animal testing both for trade and for disease control purposes is practiced.

As the disease is closer to being eliminated or when the prevalence of the disease is low, a test and cull program is required to completely eliminate it. Controlling infection in animal is the best way to prevent infection in humans. When there is infection in animals, pasteurisation of milk is an important way to reduce infection in humans. In endemic areas, vaccination is often used to reduce the incidence of infection. There is a continuous effort in development of effective vaccines for cattle and currently there are several in use. However, there are various drawbacks

such as low efficacy, interference with diagnostic test by inducing anti-LPS antibodies, being virulent for humans, causing persistent infection in vaccinated animals, having risk of virulence reversion, secretion into milk, inducing abortions in pregnant animals even at a single dose, and not having long lasting protection. Some of the live attenuated vaccines used against *Brucella abortus* are RB-51, S-19, 45/20 and SR82. There are efforts currently being made in developing more effective and promising sub-unit vaccine, vector-based vaccine and genetically engineered live attenuated vaccine. Some of the shortcomings of the currently available live attenuated vaccines are provided in the table below.

Vaccine name	Properties	Shortcoming
RB51	● Rough phenotype (does not induce anti-LPS antibody and differentiating infected from vaccinated animals (DIVA)) ● Stable ● Less virulent than S19 ● Low level of abortion	● Varying level of protection ● Infectious to humans ● Rifampin resistant
S19	● Smooth phenotype ● High levels of protection, ● 45/20; rough strain, ● SR82; limit application in some countries, similar protection to S19.	● Interference with diagnostic test ● Residual virulence, causes abortion ● Fully virulent for humans ● Reduction of milk production.
45/20	● Rough strain	● Residual virulence ● varying level of protection ● Local reaction ● Require adjuvant ● Need for repeat vaccination.
SR82	● Similar protection to S19	● Only used in limited number of countries

2.8 Geographical distribution

The highest incidence is observed in the Middle East, the Mediterranean region, sub-Saharan Africa, China, India, Peru, and Mexico. Currently, countries in central and southwest Asia are seeing the greatest increase in cases. Several countries in Western and Northern Europe, Canada, Japan, Australia and New Zealand are believed to be free from the agent.

3 Status of brucellosis in the country

3.1 Infection in cattle

In a sero-survey conducted for Brucellosis in eight government cattle farms in 2015, National Jersey Breeding Centre (NJBC), Samtse was detected with an alarming farm prevalence rate of 24.6% (28/114).

Parameters	Total sample	Positive	Negative	Prevalence (%)
NJBC	114	28	86	24.6
CRC	33	0	33	0
NHP	25	0	25	0
BSF	112	0	112	0
RMBF-Arong	55	0	55	0
RMBF-Zhemgang	69	0	69	0
NNBF	58	0	58	0
NDDC	12	0	12	0
Overall	**478**	**28**	**448**	**5.86%**

In absence of a vaccination programme, detection of sero-reactive adult animals was considered as a serious concern. All other farm animals tested negative to infection. Subsequently, a series of longitudinal studies were conducted in the affected farm that included re-testing of sero-positive animals through serological, bacterial culture and molecular tests.

Dzongkhag	No of sero-positive cases
Haa	6
Paro	2
Thimphu	1
Gasa	1
Samtse	2
Wangdue	1
Dagana	1
Bumthang	2
Trashigang	3
S/Jongkhar	2
Total	**21 (21/1099) ≈ 2%)**

The longitudinal study showed that the sero-prevalence had increased to 38% thus indicating an active infection and spread of disease within the NJBC farm. Similarly, the culture and molecular tests detected increasing number of animals shedding *Brucella abortus* organisms in milk. The Department of Livestock has taken a series of interventions to control and prevent spread of

infection. Retrospective investigations showed that the disease incursion occurred through import and introduction of new cattle into the farm. To determine the infection status at national level, the Department completed a risk-based sero-survey in 220 milk cooperatives and found 2% sero-prevalence (21/1099 cattle) in 10 out of 20 Dzongkhags. In 2018, 22 of the 33 cattle tested sero-positive to Brucellosis (sampled referred by RLDC Khangma) in Trashigang (Radi: 2 farm; Samkhar: 2 farm) including 12 cattle tested sero-positive in Norzinthang dairy farm at Trashigang Chenary.

3.2 Human infection

Five human (2 from Paro and 3 from Samtse NJBC staffs) tested positive to Brucellosis infection by Royal Centre for Disease Control, Serbithang following report of suspect cases. All infected people were treated using standard treatment regimen by the Ministry of Health.

4 Susceptible livestock population

As per the livestock statistics 2019, the number of cattle in Bhutan was 302,589, followed by goat (47,735), yak (41,918), pig (20,070), equine (16,792), sheep (11,466), zo/zom (9904), buffalo (475) and mithun (453). Further, being a bio-diverse country with abundant wildlife population, spill over into susceptible wildlife population and subsequent establishment of disease is a potential threat.

5 Diagnostic capacity

Currently laboratory services are provided through a network of 20 District veterinary laboratories (DVL), 4 satellite veterinary laboratories (SVL), 4 regional veterinary laboratories (RVL) and a national veterinary laboratories (NVL).

Test type	DVL	SVL	RVL	NVL
Staining methods	Yes	Yes	Yes	Yes
Culture	No	Yes[1]	Yes	Yes
Milk Ring Test	Yes	Yes	Yes	Yes
Rose Bengal test	Yes	Yes	Yes	Yes
ELISA	No	No	Yes[2]	Yes
PCR	No	No	No	Yes[3]
CFT	NO	No	NO	No*

[1] Available in some of the satellite veterinary laboratories
[2] Available in some of the regional veterinary laboratories
[3] Contingent to availability of the diagnostic reagent (PCR capacity is established at NVL)
**Plan to initiate at NVL*

6 Prevention strategy

6.1 Regulated import of animals

To ensure that brucellosis is not imported into the country through import of animals and animal germplasms, the import of animals and animal germplasms shall be carried out complying to the requirements set in the Livestock Rules and Regulations 2017.

6.2 Inter-Dzongkhag movement of animals

The inter-dzongkhag movement of cattle shall be strictly followed in line with the Livestock Rules and Regulation 2017. Only cattle with valid movement permit shall be allowed to be translocated from one dzongkhag to another. The concerned owner should produce health and vaccination certificate issued by the concerned livestock office along with the letter of ownership authentication issued by the Local Government to the Regulatory Authority while applying for in-country movement permit.

6.3 Intra-Dzongkhag movement of animals

The movement of animals within a dzongkhag shall be permitted without the requirement to produce movement permit. However, if there are identified households or farms with known cases of animals with brucellosis, no movement of the infected animals or other susceptible animals from the farm shall be allowed unless the farm is declared free of infection meeting the required testing protocol set by the Department of Livestock (figure 2).

6.4 Outbreak declaration and ban on movement of animal and animal products

Once the infection is confirmed in a farm, the Dzongkhag Livestock Sector through the respective Dzongkhag administration shall declare the outbreak and impose ban on movement of animals and animal products from the infected farms and other applicable farms based on risk assessment, as a pre-emptive measure to prevent spread of the disease. The Dzongkhag Administration shall lift the ban upon the advice of the respective Regional Livestock Development Centre, National Centre for Animal Health or the technical working group (TWG).

7 Outbreak response strategy

7.1 Case definition

Given vaccination against brucellosis is not practiced in the country and the current strategy is to eliminate brucellosis from the cattle population, the following case definition will be used to determine brucella infection in cattle. Until such time where NCAH is equipped with CFT, the following combination of testing will be used to confirm infection in cattle.

7.1.1 Positive/Reactor

1. Each animal testing positive using RBT during screening in the field and positive to I-ELISA at referral laboratory in subsequent testing

   Note: Cattle will also be classified as positive in the absence of significant serologic test results when other diagnostic methods, such as agent identification, result in the recovery of field-strain Brucella organisms.

7.1.2 Suspect

Cattle are classified as suspect when an animal tested positive to RBT and those animals shall be further monitored.

7.1.3 Negative

Cattle are classified as brucellosis negative when an animal is tested negative using RBT.

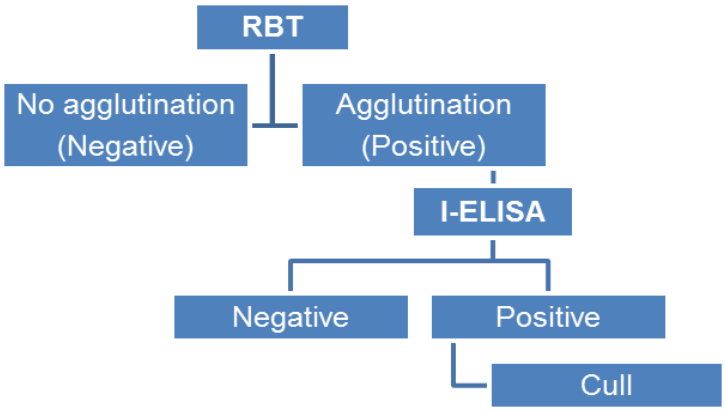

Figure 1 Algorithm for screening of cattle for brucellosis

Since the prevalence of brucellosis in the country is below 5% (2% as per the national sero-survey conducted in 2017), the outbreak response measures are aimed at eliminating brucellosis. In addition to the low prevalence, considering the shortcomings of the currently available vaccine, particularly in terms of low efficacy and risk to vaccine handlers, the country adopts **"test and cull"** strategy to respond to any outbreak. However, in the event of widespread reporting of the disease (high prevalence), vaccination programs shall be implemented to decrease the prevalence along with the **"test and cull"** strategy targeting elimination.

7.2 Outbreak detection premises

There are different places or premises in which brucellosis can be detected. In Bhutan's setting, brucellosis will be detected in Quarantine station in imported animals, private farms, Government farms and stray cattle. Responding to positive cases of brucellosis would differ

depending on where the brucellosis has been detected. Detection of positive cases of brucellosis is notifiable and it should be immediately notified using the flash reporting form.

7.2.1 Detection in Quarantine station

In the quarantine station, animal that test positive shall not be allowed to be transported into the country. Other animals in the batch will be allowed to be transported into the country with the condition that these animals are screened for three times on a two-monthly basis. It will be the Dzongkhag Livestock Sector's responsibility to coordinate screening of these animals against brucellosis.

7.2.2 Detection in Government farm

Detection of brucellosis in a government farm can warrant culling of the entire animals on the farm. However, based on the risk assessment, factoring the manner in which animals are housed and time of detection of positive cases, selective culling of positive animals can be undertaken. The farm management will be responsible to undertake the 3-D operation with the technical support from Dzongkhag Livestock Sector, Regional Livestock Development Center and the National Center for Animal Health. Upon the completion of 3-D operation, the farm management will carry out screening of farm animals as shown in figure 2, with support from DLS and RLDC, until the farm is declared of infection.

7.2.3 Detection in Stray cattle (no claiming owners)

While there will be no targeted testing of stray cattle undertaken, in the event of detection of positive cases while testing them for required reasons, the respective local government shall be responsible for managing the animal(s) with support from the Dzongkhag Livestock Sector and the Regional Livestock Development Centers. Animal thus tested positive shall be taken to the designated areas for 3-D operation.

7.2.4 Detection in Private farms

Positive and inconclusive (until final diagnosis is made) reactors will be quarantined immediately when the herd is declared infected. The entire epidemiological unit - the whole herd/farm and related herds/farms will be quarantined based on risk assessment until further appropriate action is taken. Upon detection of one or more animals positive in the farm, the process of screening and decision of action will be taken as shown in the figure 1.

The farm owners where only few animals on their farm test positive will have option to cull the entire animals or only those that has tested positive. However, given the risk of having disease transmitted to other animals that tested negative, no movement or sale of raw milk, milk products processed from raw milk, animals for reason other than to abattoir shall be allowed. Keeping in mind that brucellosis is a herd disease; it is regarded as a high-risk activity to sell

animals from an infected (positive) herd. Brucellosis may be incubated for extended periods of time, hence the sale of negative animals from a positive herd will not be allowed. The owner/farmer of dairy cattle may sell the milk if it is pasteurized or to "companies" that pasteurize the milk – selling of fresh milk is prohibited in a positive brucellosis herd.

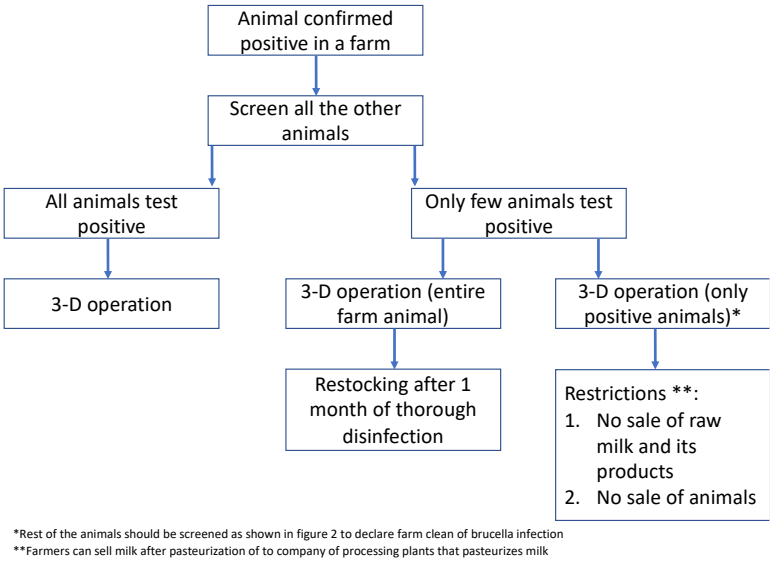

Figure 2 Schema of action to be undertaken upon detection of a cattle positive to brucellosis on a private farm

Farmers wishing to save the negative testing animals can request the Dzongkhag Livestock Sector to move the animals to a clean farm (sufficiently away from the infected farm). The clean farm has to be fenced, adequate biosecurity and management procedures be in place and all movements controlled. In such cases, testing will continue for a satisfactory period until the herd can be declared negative. The resting requirement before declaring a farm free of infection is provided in the figure below:

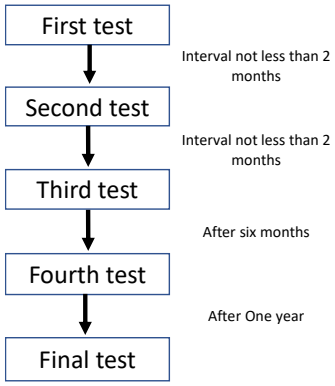

Figure 3 Testing frequency and interval required between each testing to declare farm clear of brucellosis

Neighbors of herds infected with brucellosis shall be notified & tested as a matter of priority. Investigation and testing of neighboring herds are necessary as part of the breakdown

investigation. Advice about measures appropriate to prevent or limit infection in the neighbor's herd should be provided.

All animals that test positive in private farms shall be translocated to a government premises for 3-D operation. As prescribed in the Livestock Act of Bhutan-2001, the farmers will be compensated at the compensation rate fixed by the Ministry. The Technical Working Group for brucellosis in consultation with the Livestock Production Division and Incident Operation Center will propose a compensation rate to the Ministry for endorsement. The Regional Livestock Development Center (RLDC) will lead the 3-D operation with the support from the respective Dzongkhag Livestock Sector (DLS).

7.3 Incident Command Structures and Institutional arrangements

In order to ensure that funds are mobilized and in timely manner, especially for the compensation and culler incentive payment, like in other disease outbreak response strategy e.g., avian influenza and African swine fever (ASF), the National Incident Command Committee (NICC) and the Technical Working Group (TWG) shall be activated. However, given the nature of the disease and its transmissibility characteristics, there will be no activation of incident operation center (IOC). The 3-D operation shall be undertaken as a regular outbreak response activity by the RLDCs or DLS.

The Incident Command Structure for the veterinary response to brucellosis outbreak in animals has been adapted from the National Influenza Pandemic Preparedness Plan – 2020 (NIPPP) which further is developed in line with the Disaster Management Act of Bhutan 2013 considering the requirement of highly technical and sector-based response for disease outbreaks as shown in Figure 5.

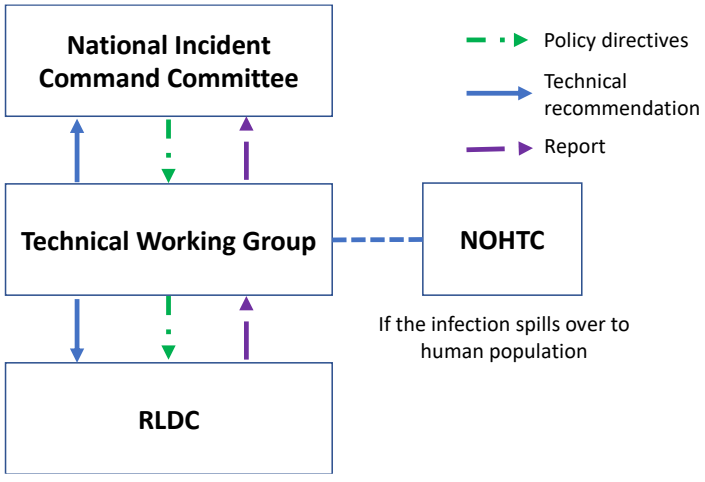

Figure 4 Flow of directives and information (reports)

In the event where control of outbreak requires involvement of the National Disaster Management Authority (NDMA), Brucella outbreak would be considered a national disaster and therefore NDMA shall be activated for smooth implementation of this plan. The NDMA shall be chaired by the Prime Minister and shall include members as stipulated in sections 7 and 8 of Chapter 2 of the Disaster Act 2013.

7.3.1 National Disaster Management Authority (NDMA) for the NBPCEP

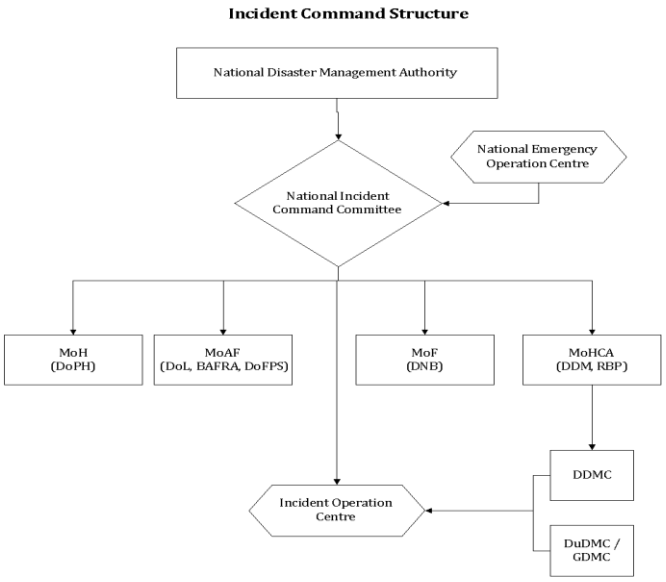

Figure 5 Incident Command Structure for NBPCEP

However, during the outbreaks of brucellosis in animal phase, NICC shall be the highest policy decision making body.

7.3.2 Roles and Responsibilities of NDMA

- Make policy decisions for the implementation of the NBPCEP as recommended by the NICC
- Mobilize required resources for the implementation of the plan as recommended by NICC
- Approve inter-sectoral lead agencies and ensure coordination amongst all relevant sectors which would be involved in effective implementation of the NBPCEP when outbreak is considered a national disaster
- Declare brucellosis outbreak as a national disaster
- Direct NICC to form committees and task forces as necessary to deal with brucellosis outbreak
- Ensure inter-sectoral collaboration and partnership with international, regional and bilateral agencies including the World Health Organization (WHO), the United Nation's Children Fund (UNICEF), the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), the World Bank (WB), the Asian

Development Bank (ADB), and the South Asian Association for Regional Cooperation (SAARC).

7.3.3 Meeting and procedures:

The NDMA shall meet once a year and as and when required. The Chairperson of the NDMA may call a special meeting, if:

- The brucellosis outbreak becomes widespread and NDMA's intervention is required
- A written request is made by the NICC; or
- The Chairperson of NICC considers necessary

A simple majority shall constitute the quorum for convening the meeting. The NDMA shall seek technical recommendations from experts within MoAF and MoH for decision-making processes. The Incident Command Structures have been adopted for proper coordination of the key stakeholders during the operation. The incident command structure will allow a smooth flow of information from the national level to the incident area and vice versa.

7.3.4 National Incident Command Committee (NICC)

The NICC is the highest policy decision-making committee for brucellosis prevention and containment activities in the country under the guidance of NDMA. It shall be responsible for providing overall guidance in the implementation of the NBPCEP both in the prevention as well as during the outbreak phase. The NICC shall liaise closely with the National Emergency Operations Centre (NEOC) for necessary support especially in the event of the national disaster and beyond. The National Centre for Animal Health (NCAH), DoL will be the secretariat to the NICC.

7.3.4.1 Members of NICC

The following members constitute the NICC:

- Secretary, Ministry of Agriculture and Forests - Chairperson
- Head, Department of Livestock, MoAF, **Deputy Chairperson**
- Head, Bhutan Agriculture and Food Regulatory Authority, MoAF
- Head, Department of Public Health, Ministry of Health
- Head, Department of Disaster Management, Ministry of Home & Cultural Affairs
- Head, Department of Forests and Park Services
- Head, Department of National Budget, Ministry of Finance
- Head, Animal Health Division, DoL
- Head, Quality Control and Quarantine Division, BAFRA
- Head, National Centre for Animal Health (Member secretary)

7.3.4.2 Roles and responsibilities in the event of an outbreak of brucellosis in Bhutan:

- Declare the outbreak of Brucellosis and issue official/public notification on recommendation of TWG.
- Authorize issuance of notice to the OIE and other trading partners.
- Take policy decisions on response and control measures based on the advice and recommendation of the TWG.
- Command response to brucellosis outbreak upon request from the TWG
- Provide policy direction for response activities.
- Issue executive orders, public notifications and press releases on disease outbreak and its containment activities.
- Facilitate and mobilize all the logistics and fund requirement for responding to brucellosis outbreaks.
- Designate media spokesperson(s) for providing disease status update, response policies and strategies, press release, etc.
- Ensure the adequacy, timeliness and relevance of communications activities.
- Maintain liaison with relevant sectors such as the Department of Disaster Management (DDM), Ministry of Economic Affairs (MoEA), Ministry of Finance (MoF), Ministry of Foreign Affairs (MoFA), Gross National Happiness Commission (GNHC), Bhutan Chamber of Commerce and Industries (BCCI), Ministry of Education (MoE), International organizations, non-governmental organizations, etc.
- Provide updates to the NDMA when required.

7.3.4.3 Frequency of meetings and quorum

The NICC meeting shall be convened within 24hours of laboratory confirmation of the disease outbreak by the NCAH. During normal times, NICC shall be convened at least once a year or as and when deemed necessary by the Chairperson. The committee will also meet as and when there are outbreaks in neighbouring/trading countries to decide on regulatory issues or when there are serious public health threats. A simple majority shall constitute the quorum and as far as possible all the members shall be present. In addition, technical experts shall be invited to the meeting.

7.3.5 Technical Working Group for brucellosis

The Technical Working Group members will comprise of experts from different sectors to advise and provide technical recommendations to the NICC, RLDC, Central farms and Dzongkhag Livestock Sectors.

7.3.5.1 Members:

S.N.	Members	Agency	Responsibilities
1	Chief, Animal Health Division	Animal Health Division-DoL	Chair
2	Specialist, Animal Health Division	Animal Health Division-DoL	Member
3	Representative from Animal Health Division	Animal Health Division-DoL	Member
4	Head, Disease Prevention and Control Unit, NCAH	NCAH-Serbithang	Member secretary
5	Head, Laboratory Service Unit, NCAH	NCAH-Serbithang	Member
6	Wildlife health focal	NCD, DoFPS, MoAF	Co-opt Member
7	Quality control and quarantine division focal	BAFRA, MoAF	Member
8	Program officer, CDD	CDD, DoPH, MoH	Co-opt Member

7.3.5.2 Terms of Reference for Technical Working Group

- Review and provide technical recommendation to the NICC on the emergencies related to brucellosis prevention and control activities;
- Apprise NICC and advise RLDC on the scale of rapid containment activities including manpower requirement, movement restriction, timeline and allocation of resources depending on the magnitude of the disease outbreak;
- Review the RLDC reports and provide timely advice to RLDC on outbreak containment activities during outbreak;
- Analyse the RLDC report and update on timely basis to the NICC
- Recommend review of NBPCEP and SOPs for brucellosis prevention and control activities
- Facilitate mobilization of resources for prevention and control activities;
- Fix compensation rate in consultation with the Livestock Production Division and RLDC for endorsement by NICC
- Carry out any specific tasks assigned by NICC

7.3.5.3 Meeting and Procedures

The committee will meet as and when required or when called by the TWG chair.

7.3.6 Role of the National Centre for Animal Health

The National Centre for Animal Health shall be the National coordinating centre for all preparedness and response activities. The NCAH shall provide necessary technical and logistic support to the RLDC while undertaking disease control measures. The NCAH shall also work as secretariat to NICC and TWG and facilitate the organization of NICC and TWG meetings as and when instructed by the Chair of NICC and TWG.

7.3.7 Role and responsibilities of Regional Livestock Development Centre

Upon receiving Executive Order from the NICC, the RLDC will initiate response measures within 6 hours. The response activities in the event of an outbreak of brucellosis will need to be undertaken as per the timeline given below.

EVENT	TIME	RESPONSIBILITY
Suspected case report by LEC/DVH/TVH		
Investigation and sample referral to NCAH	Sample should reach NCAH within 24 hours after collection	RLDC/TVH & SL/DVH
Laboratory confirmation by NCAH and reporting	NCAH should confirm the disease within 48 hours	NCAH
Convening NICC meeting	NICC should be convened within 24 hours	NCAH and DoL
Implementing Rapid Response measures including 3-D operation	Rapid respone measures within 6 hours of NICC convention	RLDC/DLS
	NICC deactivated 24 hours after 3-D operation	NICC

Figure 6 Timeline for major activities following confirmation of brucellosis outbreak

The role and responsibilities of the RLDC is as provided below:

- Overall coordination of outbreak containment activities in the respective region
- Submit activity report to TWG
- Mobilization of all logistics, supplies and human resource required for rapid response activities
- Liaison with relevant agencies within Dzongkhag/ Dungkhag/ Thromde/ Gewog level when required
- Submit budget proposals and settle all the bills
- Fully responsible and accountable for expenditures incurred during the containment of outbreak and auditing of the expenditures
- Liaise with TWG in fixing compensation rate and advocate to the farmers
- Scaling of manpower based on the scale of the outbreak
- Any other tasks assigned by TWG and NICC

7.3.7.1 Rapid response team (RRT)

The RRTs will be divided into different groups as per the mandate of the respective technical sectors involved in the disease control measures. The DoL will be mainly responsible for disease outbreak investigation, surveillance and logistic supply. BAFRA will be responsible for the 3-D operations, quarantine and movement control. The Ministry of Health will be responsible for providing prophylactic and first aid services; RBP will be responsible for the maintenance of law and order and providing support for different operations; Dzongkhag Disaster Management Committee will be responsible for providing necessary logistic support. However, all these team will be formed only if required. As a lesson from the past experience of implementing 3-D operation, rapid response teams shall be formed in ensuring rapid and effective response upon detection of brucellosis positive animals. The requirement of different rapid response teams and mobilization of human resource among the different teams under RRT will be decided by the Technical Working Group in consultation with the respective RLDC. The RRT that may be required for responding to brucellosis outbreak and their roles are as described below:

7.3.7.2 Disease Outbreak Investigation Team (DOIT)

The DOIT shall be responsible for disease investigation and confirmation of the outbreak and they shall be responsible for the identification and establishment of infected premises, dangerous contact premises, suspected premises, protected and surveillance zones. The DOIT shall also be responsible to undertake risk assessment to establish the zones and to decide on other disease control measures to be applied. Based on the requirement in the field, the TWG will deploy independent risk assessment team before the implementation of 3-D operation.

The DOIT shall consist of the following members:

- Veterinary Officer (Team Leader).
- Laboratory technician

7.3.7.3 Surveillance Team

The surveillance team (clean team) shall be involved in carrying out all necessary surveillance activities both in the protection and the surveillance zones. The team will also provide risk communication on brucellosis to the communities. The Surveillance Team shall be composed of the following members:

- Veterinary Officer/Para-veterinarian
- Laboratory Technicians
- Public health official (for advocacy)
- Forestry official during wild animal surveillance (as and when required)

7.3.7.4 3-D Team (Depopulation, Disposal, Decontamination)

The 3-D team shall be responsible for carrying out culling, disposal and decontamination of animals that has been confirmed positive to brucellosis as per the SOP provided in Annexure 2. All animals will be subjected to stamping out once a clinical disease or evidence of active infection is confirmed.

The 3-D Team shall be composed of the following members:

- Team leader: Regional Director or Animal Health In-charge (RLDC)
- One BAFRA Livestock Inspectors who will act as welfare officer, assist the team leader and coordinate decontamination
- Concerned Livestock Extension Agents: Record keeping

Cullers:

- One or two hired and trained personnel as a culler depending on number of animals to be culled
- Hired labourers for disposal of culled animals (in places where excavator hiring is not possible) and to assist in decontamination process
- One police personnel may be engaged if farmers are resistant to the culling despite the best effort by 3-D team in advocating the importance of 3-D operation

7.3.7.5 Quarantine and Movement Control Team

The Quarantine and Movement Control Team shall be responsible for enforcement of quarantine and movement control of animal and animal products from the infected farm or areas to control and prevent the spread of the disease and contain the outbreak as soon as possible. The Quarantine and Movement Control Team shall be composed of the following members

- Team leader: Regulatory and Quarantine Officer/Inspector (Livestock).
- Village Tshogpa or Mangmi

7.3.7.6 Compensation Committee

The main role and responsibility of the Compensation Committee is to ensure provision of compensation in a fair, transparent and timely manner to all eligible owners/farmers. The committee shall strictly adhere to compensation guidelines given in the document. The Compensation Committee shall be composed of the following members:

Dzongkhag level:

- Dzongdag/Gup / or a representative – Chairperson
- DLO/Geog extension officer **– Member Secretary**
- BAFRA RQO/RQI Livestock– Member
- Representative from RLDC – Member

Thromde level:

- Thrompon/ thromde theumi/representative – Chairman
- DLO/Geog extension officer – **Member Secretary**

- BAFRA RQO/RQI Livestock– Member
- Representative from RLDC – Member

8 Reporting and monitoring mechanisms

For uniform and consistent reporting, the TWG will design and provide reporting format to the RLDC during outbreak response**.** The RLDC shall submit daily updates including the minutes of meeting to TWG about the status of containment activities. The TWG shall further share information with the NICC. The TWG should monitor the functioning of outbreak response either through a report or by convening meeting.

9 Fund mobilization mechanism

The fund mobilization for the preparedness and prevention, control and elimination activities will be undertaken as per provisions of the Disaster Management Act of Bhutan 2013. In normal times, concerned agencies (DoL, BAFRA, DoPH) will propose a budget during the annual budgeting exercise for prevention activities such as awareness, surveillance, simulation exercise and capacity building activities in the concerned sectors.

In the event of an outbreak, concerned agencies will utilize the existing budget to undertake immediate disease containment activities. The RLDC will propose fund requirements for paying compensation for the animals culled and culler incentives in consultation with TWG. The TWG with notice to the NICC will forward the budget requisition to the Department of National Budget (DNB) MoF for final approval. The MoF shall release the approved budget to the concerned RLDCs.

10 Surveillance

10.1 After 3-D operation

Animals in the infected herds which had not tested positive should be kept under regular surveillance (figure 2). In the high-risk contact zones, the respective RLDC will collected a representative sample from the cattle for six months on two monthly intervals. The samples will be tested using Rose Bengal Test or Milk Ring Test. Any sample testing positive shall be referred to the National Centre for Animal Health for further confirmation.

10.2 Inter-Dzongkhag movement of cattle

Until complete elimination of brucellosis is achieved or based on the risk assessment, the risk of spread of disease is found to be negligible, the inter-dzongkhag translocation of cattle will require testing with Rose Bengal Test.

10.3 Germplasm monitoring

Random testing of imported and semen produced within the country will be tested for presence or contamination with brucella organism on regular basis.

11 Awareness education

The awareness education on brucellosis, its economic and public health impacts, transmission dynamics, prevention and control measures, current government policy of elimination, need for community support in eliminating the disease should be continuously carried out by the animal health facilities across the country.

12 Annexure

12.1 Annexure 1: Case detection and diagnostic techniques for brucella in animals

A. Objectives of testing

- To correctly identify true positive animals as positive
- To correctly identify true negative animals as negative
- To efficiently support disease control and eradication program
- To gain population freedom from infection
- To determine herd/flock prevalence of infection

B. Tests available for diagnosing Brucella infection in animals

1. **Bulk Milk Test (BMT); Milk Ring Test (MRT) and Milk I-ELISA:** This is applicable only for dairy cattle producing milk. This test is recommended for surveillance purpose to increase the efficiency of eradication policies in infected herds/flocks.
2. **Buffered Brucella Antigen Tests (BBAT), i.e. RBT (Rose Bengal Test):** This test is recommended for screening purpose to increase the efficiency of eradication policies in infected herds/flocks.
3. **Indirect ELISA (I-ELISA):** This test is recommended for screening purpose to increase the efficiency of eradication policies in infected herds/flocks.
4. **Complement Fixation Test (CFT):** This is the gold standard to confirm Brucella infection.
5. **Brucella culture and PCR for agent identification:** For confirmation of Brucella infection
6. **NH and cytosol protein-based tests:** This test is recommended in zones where subcutaneous S19 or Rev.1 vaccination is practised. This test may help in differentiating antibodies due to vaccination from those due to infection.

C. Test facilities/capacity available in Bhutan for diagnosing Brucella infection in animals

1. **Milk Ring Test (MRT):** Is a test using antigen and antibody reaction principle. The positive reaction results in agglutination with ring of cream equal or more coloured than the underlying milk. The test is very sensitive and positive samples should be confirmed by the Complement fixation test (CFT) or by an IgG ELISA. The MRT is performed on bulk milk samples to detect infection in milking dairy herd.
2. **Rose Bengal Test (RBT):** Is a test using antigen and antibody reaction principle. The positive reaction results in agglutination. The test is very sensitive and positive samples should be confirmed by the Complement Fixation Test (CFT) or by an IgG ELISA.
3. **Indirect-Enzyme linked Immunosorbent Assay (I-ELISA):** Is a semi-quantitative test using antigen antibody reaction principle. The positive reaction results in significantly higher optical density (OD) values (absorbance) compared to negative control. The test is highly sensitive and specific.

D. Diagnostic estimates

- **Sensitivity:** Diagnostic characteristic of a test to identify truly positive sample as positive
- **Specificity:** Diagnostic characteristic of a test to identify truly negative sample as negative

E. Combination of parallel and serial testing

- **Parallel testing:** Parallel testing involves conducting two or more tests on an animal or group of animals. If any of the tests are positive, the animal is considered to be affected. Parallel testing increases sensitivity and the negative predictive value, but reduces the specificity and the positive predictive value.
- **Serial testing:** Serial testing is basically the practice of running a set of tests one after another instead of as concurrent tests. Serial testing increases specificity and the positive predictive value, but reduces the sensitivity and the negative predictive value.

F. Test to be applied in Bhutan for diagnosing Brucellosis in cattle

Following testing procedure shall be applied for Brucellosis prevention, control and eradication programme in Bhutan:

1. Use a test with high diagnostic sensitivity to correctly identify true positive as positive. Rose Bengal Test (RBT) is one such test. Using such test will reduce the probability of false positive. However, due to low diagnostic specificity, there is possibility of false negative
2. Use a test with high diagnostic sensitivity as well as specificity. Indirect-Enzyme linked Immunosorbent Assay (I-ELISA) is one such test. Using such test will confirm the result of RBT and increases specificity, thus reduce probability of false negative
3. Animals will be screened in the field using RBT.
4. Suspected animals will be further confirmed using I-ELISA at the referral laboratory
5. Animal that test positive for by both RBT and I-ELISA at the referral laboratory shall be considered positive and culled
6. Culled animal shall be disposed. Appropriate samples will be collected for organism isolation and characterization

   Note: *MRT shall be used independently for regular surveillance in dairy cooperatives*

12.2 Annexure 2: Standard operating procedures for culling, disposal and decontamination

A. Purpose

The purpose of this SOP is to ensure that the implementation of culling and disposal for elimination and eradication of Brucellosis in animal species (cattle, sheep, goat and pig) are carried out smoothly, successfully within a shortest possible time to eliminate Brucella positive animals from the herd, and ultimately eradicate Brucellosis from animal population. Once the culling and disposal is completed decontamination of the premises and the disposal areas are done using the standard disinfectants.

B. Scope

This Standard Operating Procedure covers the guidelines and steps for humane culling and secured disposal of animal carcasses and infected materials (manure and farm equipment) by the culling and disposal team.

C. Materials and Equipment required

I. Personal Protective Equipment

Each culling team member must be provided with adequate protective measures to protect from infection by means of a set of Personal Protective Equipment which include:

- Goggles
- Face mask
- Inner gloves –vinyl
- Outer glover - nitrile
- Apron
- Gumboots
- Shoe cover
- Additional good quality gloves (yellow) for cullers and disposal labourers

Each person should be provided with adequate number of above PPE sets which shall be worn at all times when they are culling the identified animals.

II. Culling Equipment

- Sedation- Xylazine
- Captive bolt stun gun
- Bleeding knifes
- Plastic disposable needles and syringes
- Rope
- Water Pipes

- Buckets

III. Disinfectants

- Calcium hypochlorite (bleaching powder) or
- Calcium Oxide (lime) or
- Glutaraldehyde
- Antiseptic wash/ Hand sanitiser
- Towel
- Soap

IV. Disposal materials & equipment

- Appropriate excavating machines (based on necessity)
- Spade
- Shovel
- Pick axe
- Crowbar
- Fencing materials (Barbed wire, u-nails, poles etc)
- Plastic Sheets/Tarpaulin

D. Culling Procedures

i. General consideration

All the positive animals in the farm or infected area will be subjected to stamping out once a clinical disease or evidence of infection is confirmed. Plan for culling should be established based on the information and situation of the infected premises by the IOC Commander.

- The culling team must be led by IOC Commander supported by the Deputy Commander.
- Determine the site for culling and disposal
- To minimize the handling and reduce stress in animals, they should be preferably culled on the affected farm or site selected by the management
- The area chosen for culling and disposal is away from the dwellings, crowds, water source, religious premises, communities,
- Only individuals involved in culling operations are in the area.

Identify and establish a proper site for culling near the disposal site decided by the team. The site should be as far as possible accessible to road for easy transportation and burial. The culling team will put the PPE set as per the prescribed SOP before entering the site of operation. The members are designated specific tasks to be perform in the orderly manner along with the facilities for fast culling and disposal procedures. The Team Leader shall then provide necessary briefing to all culling and disposal groups.

The SOP for culling is as follows:

- Tie the infected animals with identification.
- Put on PPE as per the SOP for use of PPE before crossing the culling, cleaning and disinfection line (protected zone).
- Cull the animals as per the culling procedures
- Dispose the culled animals into the pit as per the pit specifications and locations prescribed as per Sop.

ii. Restraining of animals

Animals are properly tied and restrained for stunning near the disposal site. Once the animal is properly restrained, animal is sedated using Xylazine and observe for minimum of 10 minutes before euthanasia of the animals.

iii. Euthanasia

Animals are euthanized using the captive bolt as per the specifications mentioned below. The captive bolt stun gun is placed on the forehead as per the Fig 1 and euthanized as per the instruction manual of the equipment. Once the animal is knocked down using the captive bolt, the Jugular vein of the animal is severed for bleeding. Once the animal is declared dead by the competent person, it is dumped into the disposal pit.

E. Culling Method

The method chosen for euthanasia of animal must be safe, humane and efficient.

I. Captive Bolt Stunning Techniques for Cattle

A captive bolt stunning gun kills the animal by instantly making the animal unconscious without causing pain. A captive bolt gun has a steel bolt that is powered by either compressed air or a blank cartridge. The bolt is driven into the animal's brain. It has the same effect on the animal as a firearm with a live bullet. After the animal is shot, the bolt retracts and is reset for the next animal. A captive bolt gun is safer than a firearm. There have been some questions about whether or not a captive bolt actually kills an animal. Practical experience in slaughter plants indicates that cattle shot correctly with a penetrating captive bolt have irreversible damage to their brain and they will not revive. If a non-penetrating captive bolt is used the animal may revive unless it is bled promptly.

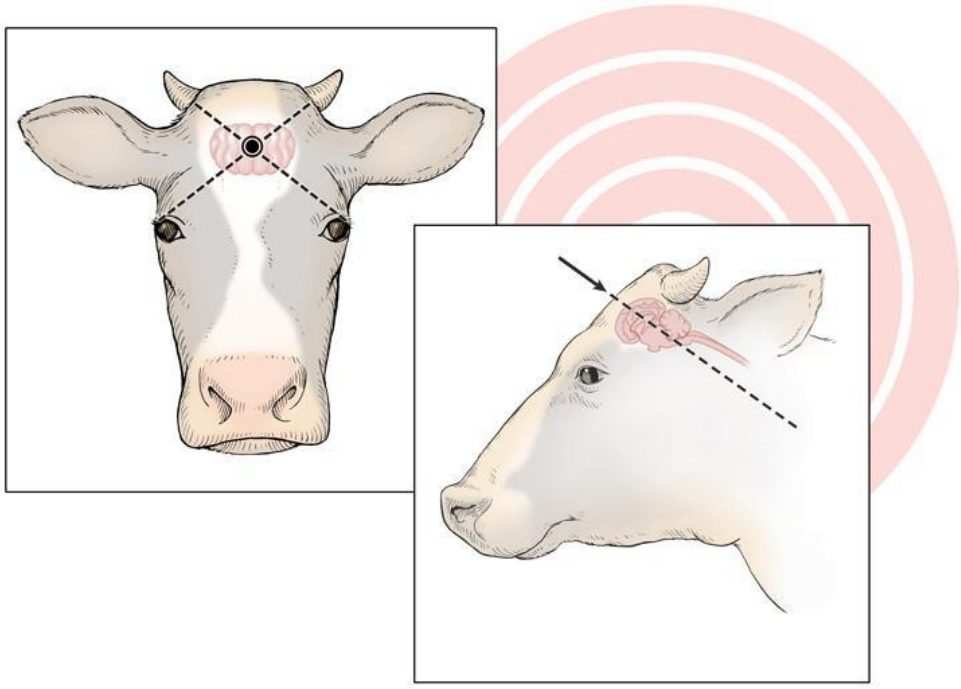

Fig1: Appropriate location for captive bolt or gunshot in cattle. Reprinted with permission: J.K. Shearer and A. Ramirez, College of Veterinary Medicine, Iowa State University www.vetmed.iastate.edu/HumaneEuthanasia (2013)

When euthanizing using stun gun, the gun should be aimed perpendicular to the intersection of two imaginary lines, each drawn from the outside corner of the eye to the center of the base of the opposite horn (or where the horn would be on dehorned animals) – not right between the eyes (see photo 1 of figure). The target site and angle are important to ensure immediate unconsciousness of the animal is achieved to minimize pain and distress. The gun should be approximately 30 to 60 centimetres (12 to 24 inches) away from the head. Never hold a gun flush against the head. This could cause the barrel to explode, harming the operator. Proper restraint is necessary to ensure you hit the target the first time. Restraint can include the use of a halter, chemical sedatives, or a livestock chute or gates. The restraint chosen will vary depending on the age of the cattle, disease process, and temperament and behaviour of the animal.

There are two types of captive bolts, penetrating and non-penetrating. Penetrating captive bolts go through the skull to physically damage the brain. Non-penetrating captive bolts do not enter the brain, but cause unconsciousness due to the force of the bolt against the head. Penetrating captive bolts are appropriate for use on cattle of all sizes, but non-penetrating captive bolts are less reliable on larger animals, so can only be used for young calves. Ensure you select the correct cartridge for the weight of the animal you are euthanizing. Consult the manufacturer's instructions for more details. When in doubt, select the heavier cartridge.

The target site for captive bolts is the same as the target site for gunshot. Unlike guns, captive bolts need to be held flush against the head when discharged. Therefore, proper restraint of the head is necessary. The animal can be standing or laying in any position that allows you to hold the captive bolt against the head. Once stunning with a captive bolt has been performed, trained personnel must ensure that the animal is unconscious before applying a secondary step.

- **Signs of unconsciousness**

Once you have applied a method of euthanasia, immediately check for signs of unconsciousness. Some signs that indicate that the animals is unconscious include:

- Lack of natural rhythmic breathing; If the animal was standing, it collapses and does not attempt to right itself. If the animal was already lying down, its muscles will relax and its body will go limp;
- Muscles become rigid immediately after the shot, followed by uncoordinated movements, such as involuntary muscle contractions, only (no coordinated movement); Lack of jaw tone; Eyelids remain open with eyeballs facing straight ahead; Lack of corneal reflex or 'blinking response' which can be tested by touching the surface of the calf's eye, if the calf does not blink that means there is no corneal reflex.
- Two or more signs should be present to confirm the animal is unconscious. If the animal begins vocalizing, attempts to right itself, lifts its head, or has a corneal reflex, it is conscious. If this occurs, immediately reapply the method of euthanasia, or use a backup method.

- **Secondary step (for non-penetrating captive bolts only)**

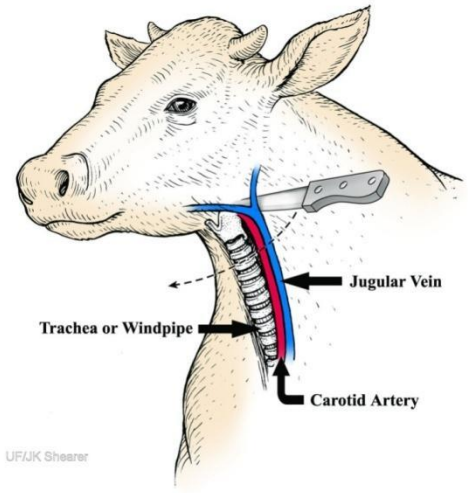

Fig 2: Exsanguinations by severing the jugular vein, carotid artery, and windpipe. Reprinted with permission: J.K. Shearer and A. Ramirez, College of Veterinary Medicine, Iowa State University www.vetmed.iastate.edu/HumaneEuthanasia (2013)

Once the animal has been rendered unconscious by the captive bolt, bleeding can be performed by severing the carotid arteries and jugular veins located on either side of the neck (see fig 2). The windpipe should also be severed at this time. When the animal is being bled out, you must ensure that all the blood is carefully collected so that it does not contaminate the environment. Once the blood is collected it must be properly disposed as the blood of an infected animal can spread disease throughout the herd. An alternate secondary method is to pith. Pithing involves placing a rod or pithing tool into the hole created by the penetrating captive bolt and moving it around to further destroy the brain.

- **Death**

Death may not occur immediately, but the animal must remain unconscious until death. You must confirm the animal is dead before attempting to move it. Indicators that can be used to assess whether the animal is dead are:

- Lack of a heartbeat (using a stethoscope placed under the left elbow) and signs of respiration for at least five minutes
- Both these signs should be present to confirm death, with the absence of any signs of the calf regaining consciousness.

II. Chemical method

- **Restraining of animals**

Animals are properly tied with ropes or halter and restrained for sedation.

- **Sedation with xylazine**

Prior sedation with xylazine is used @ 0.4 mg/kg bodyweight intramuscularly. But as per field experience and the xylazine that is currently available (20 mg/ml) with the veterinary centres, at least 1 mg/kg body weight is required. Once xylazine is administered, wait for about 10 minutes for the animals to get sedated and calm down. The animal becomes sternal recumbent on the ground once fully sedated.

- **Euthanasia with saturated solution of magnesium sulfate**

Magnesium sulphate ($MgSO_4$) also known as epsom salt, in solution was one of the first anaesthetics and euthanasiates for large animals before more modern drugs were developed. It does not present the carcass disposal problems of barbiturates, and is safer for the operator and more humane than intravenous potassium chloride, which causes heart attack without first diminishing consciousness.

- **Preparation of saturated solution of magnesium sulfate**

Saturated $MgSO_4$ solutions are prepared by adding the salt to a container of water until it no longer dissolves. The saturation point is reached when vigorous stirring does not remove the salt crystals at the bottom of the container, or simply add 2 kg of $MgSO_4$ to an empty 5 L container and top up with water. Solubility of $MgSO_4$ in water is between 300 to 400 gm/L over the 10-30°C temperature range (Table 1). Depending on the ambient temperature, consider the amount of $MgSO_4$ to be added to prepare saturated solution. Similarly, if heat is applied to enhance solubility, consider the proportionate amount of $MgSO_4$ to be added.

Table 1: Solubility of $MgSO_4$ in water at different temperatures. Source: https://en.wikipedia.org/wiki/Solubility_table

Temperature (°C)	0	10	20	30	40
$MgSO_4$ solubility (g/100 mL water)	25.5	30.4	35.1	39.7	44.7

Volumes as small as 500 mL of $MgSO_4$ may kill a 500 kg xylazine-sedated cow, however, sometimes 50% more volume is required. Therefore, the recommendation is to be prepared to administer 2 mL/kg of the $MgSO_4$ solution. In practice, the solution is administered until death occurs.

- **Steps of euthanasia with SS-MgSO4**

Once the animal is recumbent or sufficiently sedated with xylazine, the saturated solution of Magnesium Sulfate (SS-$MgSO_4$) is administered intravenously until the animal is dead. Death is achieved consistently with dose rate of 2 mL/kg bodyweight of SS-$MgSO_4$, although half of dose rate will kill most livestock. When the SS-$MgSO_4$ is gravity administered via an elevated bag or bottle, the transition to death is smooth, within 3-5 minutes (Figure 1). There is little or no leg, body or head movement and no outward signs the animal is distressed. The needle that fits into jugular vein should be at least 12-14 gauze to facilitate smooth flow of SS-$MgSO_4$. In situation where the ambient temperature is low to allow crystallization of solution, a 20 mL or 50 mL syringe may be used to draw SS-$MgSO_4$ and administer at slow pace through the jugular vein.

Figure 1: SS-$MgSO_4$ administered to a cow with gravity method

Once the animal is declared dead then it can be safely buried. To declare the animal is dead and lack any signs of life, refer the Section 5.6 and 5.7.

- **Death**

Death may not occur immediately, but the animal must remain unconscious until death. You must confirm the animal is dead before attempting to move it. Indicators that can be used to assess whether the animal is dead are:

- Lack of a heartbeat (using a stethoscope placed under the left elbow) and signs of respiration for at least five minutes.
- The onset of death is also determined by the fibrillation of heart. With the use of stethoscope listen to the fibrillation of heart as soon as the animal is euthanized.
- Both these signs should be present to confirm death or progressing to death, with the absence of any other signs of the animal regaining consciousness.

F. Disposal of animals in disposal pit

The first choice, by far, would be on-site burial. Identify the site for burial such that wild animals, dogs and human beings cannot access the carcass once buried. Considerations include the amount to bury, site availability, soil type, water table, nearby wells or ponds and digging equipment available.

G. Selection of disposal site

The most important aspects in the site selection is the isolated nature of the site which is well-protected from people and scavenging animals. In some occasions it may be necessary to guard the site for few days to avoid the risk of carcass removal by wild animals and human beings.

H. Important considerations for burial site selection include:

- Access to the site – for both equipment to dig the burial pit and for the delivery of livestock, carcasses or other materials to be buried.
- Environmental-distance to water sources, bores and wells; height of water-table; proximity to buildings, especially houses; proximity to neighbors or public lands including roads; slope of the land drainage to and from the pit; permeability of soil; sufficient space for temporary storage of overburden; and direction of prevailing wind.
- Construction considerations-avoid rocky areas (slows digging and increases costs) but select soils with good stability capable of withstanding the weight of equipment used for construction of diversion banks if required. Similar banks should be constructed to prevent any liquids escaping from the burial site.
- Fencing is necessary to exclude animals until the site is safe for use.
- If government land is not available leasing/compensation for private land for the disposal pit for the next 3 years should be considered after negotiation depending on the emergency situation. In city area if the disposals of the animals are undertaken, the

selection of the site should be done jointly with the city corporation and the necessary approval sought from the competent authority.

- **Site selection**

Important considerations for selecting burial sites are:

- access to excavators/JCB for digging the burial pit and also for the delivery of livestock, carcasses or other materials to be buried;
- Distance to spring water and dwellings
- Height of the water table
- Away from buildings and houses
- Isolated place and away from the main highway
- Sloppiness of the land and the drainage
- Impermeable soil to avoid the leakage to nearby areas'

- **Construction considerations:**

- Easy to dig
- Stable soil for digging and for landing the excavators and unloading the carcasses or animals.
- No seepage of the carcass into the public places and highways
- Fencing provision using the locally available material

- **Disposal of carcasses**

As far as possible the culling of animals should be done at the site of disposal in order to avoid the contamination of the premises and also the transportation route during transportation. The live animals transported should be properly tied in the truck and no halt on the high way done. All the feeding and watering is done inside the vehicle. The floor of the truck should be with enough bedding or flooring materials to avoid the leakage of the contents during transport.

- **Burial pit construction**

The dimensions of the burial pit will be determined by the equipment used, site considerations and the volume of material to be buried.

- **Pit dimensions**

There are no standard dimensions for the disposal of the carcasses. But the main approach for disposal is to avoid the excess to wild animals and human beings thereby to avoid the spread of the disease and also the smell during the process of decomposition. The base of the pit must be at least 1 m above the water table. Allow a fill capacity of about 1.5 m^3 for each adult beast or 5 adult sheep. At least 2 m depth of soil is required to cover carcasses to ground level. The standard dimensions of disposal pit for Cattle for Brucellosis or other diseases can be as follows:

The pit size of 3 m wide and 5 m deep can be filled with carcasses to within 2.5 m of ground level. This will accommodate 5 adult cattle per linear metre (3 × 2.5 × 1 = 7.5 m^3; 7.5/1.5 = 5 cattle which is almost equivalent to 25 adult sheep) as shown in Fig 3.

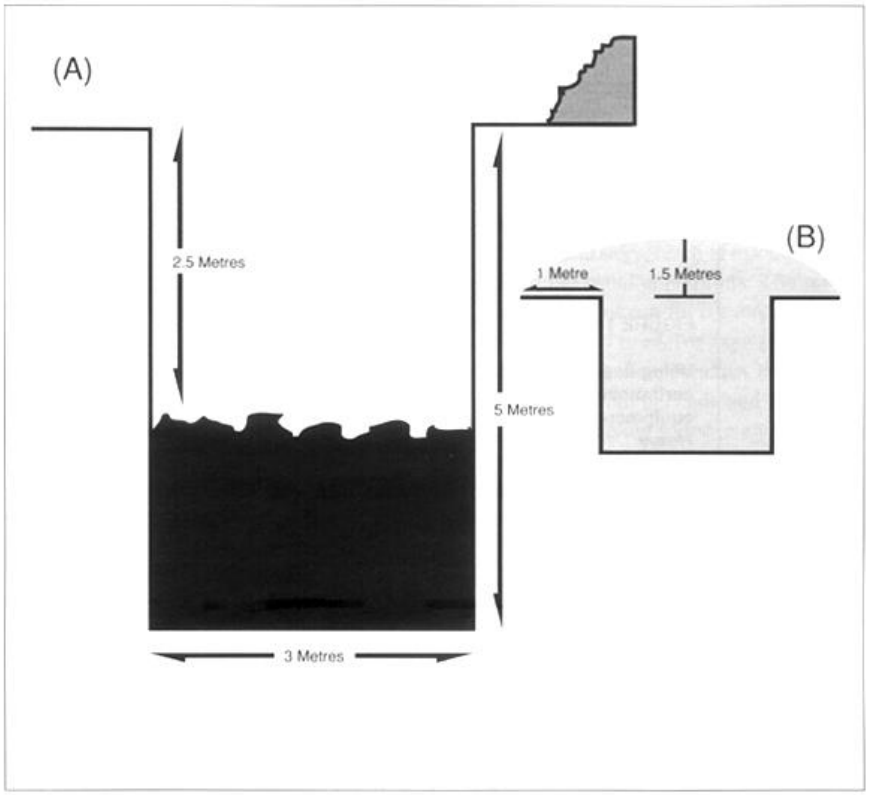

Fig 3: Disposal of carcasses by burial; (A) open pit, (B) freshly closed pit

The dimension of the pit will be decided by the number of animals to be disposed and the area of culling including the geographical terrain. Make sure that no animals are alive when dropped into the burial pit. If this happens, animals must be humanely killed. Carcasses should be covered by about 400 mm of soil and then an unbroken layer of slaked lime {$Ca(OH)_2$}. If this lime is applied directly to carcasses the decomposition process will be significantly delayed. When closing the pit, surplus soil should be heaped over the pit as overfill. The weight of soil acts to stop carcasses rising out of the pit due to gas entrapment, prevents scavengers digging up carcasses, helps filter out odors and assists in absorbing the fluids of decomposition. After pit subsidence it will be necessary to replace any topsoil not utilized during pit closure. The refilling of the pit should be undertaken as and when required when the compression of the soil leads to space in the pit.

Disinfectants are needed to be sprayed in and around the farm premises, culling and disposal sites in and around the pit. The burial pit should be located away from human and animal living

areas and water–including wells, lakes, ponds or rivers. The burial pit should be large enough to hold all of the carcass and at least 0.6 meters (2 feet) of soil on top of the carcasses.

- **Protection of disposal pit**

All the disposal pit should be properly fenced using the iron poles and barbed wires depending on the field situation. In case of remote areas the use of wooden poles and barbed wires may be explored to reduce the cost of fencing.

The main objective for disposal of carcasses, animal products, materials and wastes is to prevent the spread of the infection. This process is one of the important steps in the emergency animal disease eradication programme for Brucellosis, particularly when a stamping-out policy is followed with the strategy of culling and disposal approach. Disposal should be completed as soon as possible after euthanization in order to minimize the spread of the disease through the dispersion of infectious material by carnivores or human beings. It is also easy to dispose the carcass when fresh. Carcasses and relevant infectious materials waiting for disposal should be under strict supervision to avoid unauthorized access and to prevent domestic pets, wild animals from removing potentially infectious material. If disposal is delayed, carcasses should be thoroughly sprayed with an approved disinfectant.

- **Organizing stamping out**

The IOC Commander should brief the members on the task regarding the number of animals and their owners. The process may involve either individual or whole communities in the area. The disposal teams are aware of the zoonotic nature of the disease and its consequences. The use of N-95 mask and the minimum PPE sets are worn before the disposal of the carcass.

- **Earthmoving equipment**

The preferred equipment for digging burial pits is an excavator, which is the most efficient for the construction of long, deep pits with vertical sides. The advantages of using this machine is to store topsoil separately from subsoil for refilling the pit after disposal of the carcasses. The equipment can be used to fill the pit with carcasses or other materials and close it without disturbing the carcasses. In case of non-availability of the excavators, manual digging of pit may be done. Burial pit construction. The dimensions of the burial pit will depend on the equipment used, site considerations and the volume of material to be buried. Pits should be as deep as possible with vertical sides.

When closing the pit, surplus soil should be heaped over it as overfill. The weight of soil prevents carcasses from rising out of the pit because of gas entrapment, prevents scavengers digging up carcasses, helps filter out odors and assists in absorbing the fluids generated during

decomposition. After pit subsidence, it will be necessary to replace any topsoil not utilized during pit closure.

- **Gas production**

Gas produced by decomposition within unopened carcasses may result in considerable expansion of the buried material, to the extent that the surface of the closed pit may rise and carcasses may be expelled. It is recommended that large animal carcasses should be opened by puncturing the rumen from the abdominal cavity.

- **Covering buried carcasses with lime**

The carcasses are covered with lime to protect the carcasses from being uncovered by carnivores and earthworms. It also helps to prevent earthworms from bringing contaminated material to the surface after pit closure. Once the carcasses are inside the pit, cover with soil up to 400mm followed by an unbroken layer of slaked lime - $Ca(OH)_2$ - before filling is completed. It is not recommended to directly apply lime on the carcass as it may slow down or prevent decomposition.

- **Site inspection**

The site should be monitored daily for about three days followed by regular monitoring at intermittent intervals which can be decided by the management. The main reason for site inspection after burial is to see that the carcass are all intact in the soil and not removed from the pit. Any content during the decomposition stage will reduce the volume and so there is change that the pit becomes unfilled which may become trap to other animals. The pit need to be refilled in case the materials in the pit has settled at the bottom.

- **Steps to be followed after Culling and Disposal**

Culling and disposal team members should remove PPE and place them in trash bag, which are to be placed in biohazard plastic bags and either burnt or disposed along with the carcass before leaving the site.

  - By the end of each work day, culling and disposal team members shall dump all the used PPE and other infectious materials.
  - All shall disinfect shoes, thoroughly wash hands at the wash station and sanitize their hands.
  - All tools and other equipment must be cleaned and disinfected before taking to the farm premises or dwellings
  - All personnel must disinfect their foot by dipping them footbath before leaving the place.
  - Similarly all parts of vehicles (especially tyres) must be disinfected at culling and decontamination line.

- Once the personnel protective equipment has been removed, designated personnel must disinfect personal footwear.
- Personnel may not re-enter the infected premises without following the requirement for entering the infected premises.

- **Personal Safety**

During the disposal of the animals, utmost care is taken to avoid injuries to the team. There should be enough first aid and emergency services on standby during the operation. The following safety measures should be adhered strictly:

- All individual involved in culling and disposal operations should be provided with appropriate PPE and training on how to properly use them.
- All the members should be treated with appropriate medicines or first aid before entering infected area.
- It is recommended that, if possible, all people exposed to infection or operations should be monitored by local health authorities at least for 7 days.
- If symptoms of Brucellosis are detected during or after the operations are over, there should be a clear way to report this information to local health officials.

12.3 Annexure 3: Standard operating procedures for decontamination

A. Purpose

The purpose of developing this SOP is to ensure that all decontamination procedures are carried out smoothly, effectively and successfully post disease outbreak/culling and disposal completion. Decontamination means removal or neutralization of infectious agent (Brucella organism) through process of cleaning and disinfection. The purpose of decontamination is to ensure that live bacteria does not remain and re-emerge on the premises after culling of animals. Thus, cleaning and disinfection is a vital component of the decontamination team.

B. Scope

This SOP describes guidelines and steps to be followed for effective decontamination by the team.

C. Materials and Equipment Required

I. Personal Protective Equipment

Each member shall be provided with adequate protective measures from infection by means of Personal Protective Equipments (PPE) which include:

- A coverall (Enviroguard with hood and boots)
- An N-95 respirator
- Goggles (chemical splash)
- Outer gloves – (Nitrile, Size 10, 11-mil)
- Inner gloves – (Vinyl, 4 mil)
- Shoe covers (DuPont Proshield III)
- A plastic apron that comes in a pouch
- Rubber boots
- A Respirator Fit Test Kit (with Bitrex solution)
- Hard hat with face shield

Multiple sets of PPE will be necessary to allow for workers to take breaks. All the 3D members involved in the operation should use gumboots and thorough disinfection while moving from one house to the other.

II. Disinfectants

There are different disinfectants of choice for the disinfection of different materials and surfaces against Brucella organisms as shown in the table below

Disinfectants					Neutralizing agents	
Classification	**Active ingredients**	**Recommended concentration**	**Recommended contact time**	**Optimal application**	**Active ingredients**	**Concentration**
Aldehydes	Glutaraldehyde	4%	20 min	Equipment, goods	Glycine	2%
Halogens	Sodium hypochlorite	2 g/L	20 min	Biological material, smooth surface	Sodium thiosulfate	0.2%
	Trichloroisocyanuric acid	4 g/L	30 min	Lab environment, Medical supplies	Sodium thiosulfate	0.4%
Quaternary ammonium compound	Benzalkonium chloride	0.2 g/L	/	Skin, mucous membranes	Tween-80 + Phosphatidylcholine	0.5% + 1%
Phenolic	Lysol	10 g/L	30 min	Object surface	Tween-80	1%
Alkaline	Sodium hydroxide	10 g/L	/	Field, animal housing	Hydrochloric acid	10%

"/" indicate there is no recommended contact time.

III. Decontamination supplies

- Hand-operated and power sprayer (3000 PSI) used to dispense disinfectant.
- Alcohol wipe
- Foot bath with tray and mat

IV. Other supplies required

Maintenance tools (screwdrivers flat and Phillips, hammer, adjustable wrench, crowbar, and scrapers)

V. Personal cleaning and disinfection supplies

- A scrub brush for removing dirt and other particles before using disinfectants.
- Four bars of soap that you can use to wash your hands and face.
- A plastic basin that you can use to create a foot bath.
- Shovel
- Spades
- Biohazard bags and sacks

VI. Biohazard control materials

A few alcohol pads, 70%ethanol - these are generally used to wipe your hands after removing your PPE. These items should be worn at all times when they are near the infected animal or infected premises.

D. Method - Decontamination

Adequate cleaning and disinfection of infected premises requires planning before the culling occurs as well as work after depopulation to effectively remove the organism. Particular attention should be paid to the decontamination of shed as the organism can survive in the environment for several months at different temperatures and conditions. Contaminated fomites, such as clothing, footwear, crates, feed sacks, milking utensils and other equipment should be decontaminated, if possible. People should undergo personal decontamination procedures. Decontamination should include standard insect vector and rodent control to minimize mechanical spread of the agent to nearby premises.

iv. General consideration

Identification of a decontamination site - Cleaning and disinfecting activities of infected premise should be limited to areas inhabited by or exposed to animals and attendants. The team leader should evaluate each premise with this objective in mind and make a reasonable determination as to whether materials can be effectively cleaned and disinfected or should be discarded. The efforts to keep the pasture land un-grazed for few months can also be explored with rotational grazing methods.

Materials fall into three categories:

- Structures: Sheds
- Clean the shed effectively and disinfectant once daily for about a week.

v. Decontamination procedures

Preparation for decontamination

Identify and establish a proper site outside and close to periphery of the culling and decontamination line for putting on PPE, unloading materials and equipment required for decontamination. Where the infected area is accessible by road, a decontamination crew vehicle shall be parked at this site. Take off all materials and equipment from the vehicle.

Before entering the infected premises

Assemble the team and organize into groups as per the specific tasks to be performed in the orderly manner and distribute the materials and equipment to each member. The Team Leader shall then provide necessary briefing to all decontamination groups. Put on PPE as per the SOP for use of PPE before crossing the culling and decontamination line (protected zone). Decontamination team shall be divided into groups – the first group should start decontamination in the infected farms and other group(s) shall start decontamination from the periphery of protected zones and move towards centre of the infected area. Once personnel have entered premises, they may not cross back over the culling and decontamination line for any reason without removing and properly disposing of all PPE and proper personal disinfection.

Groups identified for decontamination of the infected farms shall only come out after completing their task. The decontamination team should allow the culling and disposal team to complete their task and then only start their operation. Prepare the select appropriate disinfectants as recommended in the Table I. It is important to wear PPE when mixing disinfectants because it can irritate the skin and eyes.

vi. Clean-up

The aim is to remove, without using water, all manure, debris, feed, etc, to expose surfaces for a second round disinfection. This is very important as organic material reduces any disinfectant effectiveness. All structural surfaces must be cleaned of any dung, splashing of blood, secretions and other contaminated materials. The next step is a wash down with a low pressure sprayer using a detergent or bleaching powder.

vii. Full scale disinfection

Disinfectant to be sprayed in the following order – roof, walls and finally the floor. Inspection must be carried out to ensure that everything has been completed- repeat clean -up and disinfection if there is doubt. Another round of full disinfection 7 to 14 days later. Final disinfection before restocking should be carried out.

viii. Decontamination of equipment used for decontamination

The other consideration is the decontamination of contaminated equipment used. The primary concern would be for anything used during stamping out. This would include items like:

- Excavators,
- vehicles,
- Shovel and spades
- Crowbars' etc.

If any trucks, vehicles, motor cycles, etc are on the contaminated site they must be decontaminated before leaving the premise. All under parts and wheels of cars should be sprayed with water and disinfection.

ix. Personal decontamination

The following procedures will apply to ALL personnel before leaving an infected area any quarantined area which is grossly contaminated with the disease organism. Culling and disposal team members walk to the cleaning and disinfection line and remove PPE and place in trash bag, which are to be placed in biohazard plastic bag.

- Industrial hard hats must be scrubbed and set aside.
- Hands must be washed in disinfectant and scrubbed.
- Warm soapy water is recommended for washing face, hair, skin, etc. Alternatively, the pH of the washing solution can be raised (by adding sodium carbonate) or lowered (by adding citric acid) to enhance antiviral action.
- Disposable gloves must be decontaminated before discarding and reusable gloves are to be decontaminated before reusing.
- Plastic overalls - use a sponge or low pressure pump and wash the overalls from top to toe to remove gross material paying particular attention to the back, under the collar, zip and fastenings and the inside of pockets.
- Boots and shoes should be scrubbed down, particular attention being paid to the sole.
- The person then walks across the area, washes feet in a footbath, changes into clean overalls and street shoes and leave directly without re-exposure to contaminated areas.
- The plastic bags containing used overalls and other articles are sealed and given a second wash down in disinfectant and then either buried/burnt or taken for cleaning. These garments should be autoclaved or treated as contaminated clothing in a hospital laundry.
- On returning to home or lodgings, the person should have a long hot bath or shower.

x. Personal Safety

All individual involved in decontamination operations should be provided with appropriate PPE and training on how to properly use them.

xi. Procedures for Handling Disinfectant

1. Store powder tightly in closed plastic container in a cool, dry place. Ensure that the area where it is stored is secured and cannot be accessed by authorized persons.
2. Follow instructions on the label for disposal.

12.4 Annexure 4: Compensation payment modalities

A. Background

The containment and spread of Brucellosis is determined by early detection and culling methods; wherein culling of animals becomes mandatory to stop further spread of infection to healthy herd. However culling of positive animals in the field would depend critically on payment of incentives to the owners. Without adequate compensation arrangements in place, dairy farm owners will have no incentive to dispose positive animals that may result in the affecting healthy herd and pose threat to human health. Therefore, it was felt essential to institute a mechanism within the Government to compensate the affected herd owners from mandatory culling and inform them in advance about the availability of such fund. The Technical Working Group for brucellosis in consultation with the Livestock Production Division and the respective RLDC will determine and fix the rate per animal. The team shall be guided by following guidelines.

The guideline intends to provide information on the operational aspects of the compensation fund with an aim to ensure quick and fair financial compensation to the affected herd owners in the event of mandatory culling of the positive animals to Brucellosis. *The Livestock Act-2001 under its sub-para 9.3 clearly states that "the Government has the authority to compulsorily destroy animals, animal products or feeds or any consignments that it considers to be risky and pay compensation as prescribed by the Ministry"*.

B. Objectives

The main objective of this guideline is to outline the operationalization and payment procedures for compensation for culling Brucella positive animals in the country.

C. Qualifying criteria for compensation payment

The farms in the affected areas will be assessed for the compensation payment.

- Both infected and at-risk uninfected animals culled as a measure to prevent the spread of disease

D. Determination of compensation rate

The Technical Working Group for brucellosis in consultation with the Livestock Production Division and the respective RLDC will determine and fix the rate per animal. The following criteria will be assessed for valuation of compensated animals.

1. Age
2. Body weight
3. Market value

4. Production stage
5. Cost of production

E. Disbursement mechanisms of compensation fund

The NICC shall approve the compensation payments as and when proposed by the compensation committee through the Incident Operation Centre (IOC) and should make payment to the affected owners within one week of culling.

F. Role of compensation committee

The main role of compensation committee is to ensure and facilitate the payment of compensation in fair, transparent and timely manner to the affected household/owners.

G. Responsibilities of compensation committee

- Will verify and approve the list of affected herd and animals eligible for compensation in the villages/farms.
- To make payments to the eligible affected herds in fair, transparent and timely manner.
- Compile the records of the culled animals and owner details.
- Review the market value of the cows and fix the compensation rates in line with Priority Sector Lending Scheme
- Get proof of payment from the recipient of the compensation rates.
- Submit completed documents to the RLDC for payments made as per the compensation format

H. Mode of payment

The compensation committee will collect copy of slips (forms) from the 3-D team. The details in the slip should be entered in the summary culling record and individual herd owner forms (designed by RLDC). The owner should produce slip to the compensation committee and payments will be processed after cross checking by the committee. The committee will process the payment using two forms and submit completed documents to the RLDC. Payment to the herd owner shall be done electronically.

12.5 Annexure 5: Standard operating procedure for collection of blood for serological testing and milk for serological and bacteriological testing for brucellosis

A. Introduction

Serology is available for a range of bacterial and viral diseases as well as for some Chlamydia, Mycoplasma, and rickettsial, protozoan and metazoan diseases. A serological test is used to show the presence or absence of antibody to the specific etiological agent or group of agents. The presence of antibody indicates exposure of organism, which may be due to current clinical condition or to earlier unrelated infection.

In tests where result are expressed in titre, the best evidence of infection is the demonstration of a four-fold titres rise between samples collected early in the clinical episode and those collected 2- 3 weeks later. A titre variation of less than four-fold (one dilution) is within the normal variation of serological tests and is not significant.

Since many animals in endemic areas may have antibody to given organism, a single sample from in affected animals may not allow the serological test to interpreted. Single serum sample is particularly useful in eliminating a diagnostic possibility.

Frequently, presence of antibody in the acute phase or absence in the convalescent phase will eliminate a diagnostic possibility. Haemolysed or contaminated samples often give unreliable results in some serological tests. Poor quality samples will give poor quality result, necessitating retesting of the animals involved.

B. Application and scope

In order to increase efficiency and to facilitate processing, examining, and reporting of Brucellosis surveillance serological submissions, this SOP shall be implemented by all veterinary surveillance team.

C. Objective

To guide through the process of collection good quality blood

D. Apparatus/equipment

- Blood vacuum tubes, 10ml. These should be silicone coated, without anticoagulant
- Sterile screw-capped, 5ml for serum samples
- Needle
- Syringe
- Needle adapter
- Gloves

E. Procedure

I. Preparation of serum

Collect the blood in a tube from the living patients. It should not be agitated and kept in a cool place. Dry syringes are to be used for collection in order to avoid haemolysis. The amount of blood to be collected is approximately 5 ml in large animals.

Keep the tube in an angle. Clotting of blood will take place in 1-2 hours and serum separation depends upon the temperature and it is separated in about 2-24 hours and a clear serum appears in the tube.

Pipette out the serum in a serum vial. Stopper it and after labelling, dispatch to the lab with forwarding letter.

Note: *While preparing serum, care should be taken to prevent haemolysis. Otherwise, the serum becomes useless and unfit for tests*

II. Collection of serum samples

Blood samples should be collected aseptically, using a 10ml blood vacuum tube. At least 5ml of blood should be collected. When a range of serological tests is required (particularly when viral serology and non-viral serology is to be undertaken), duplicate samples should be collected. Blood and faecal materials should be removed prior to dispatch, to reduce the risk of contamination of laboratory staff the specimen. Use a separate sterile needle to avoid mechanically transmitting infectious agents from one animal to another.

III. Labelling samples

Samples must be labelled serially (1- 30) with a waterproof pen, preferably on an adhesive label. Keep a key list, which correlates sample number with animal identification. Do not label the stopper, which is removed during testing.

The specimen advice from submitted with the samples should list clinical details beside each sample numbers. This will allow the laboratory to offer an informed comment on the results and perhaps apply other relevant tests.

Do not label samples with tag numbers, names, etc., as this leads to confusion and errors in reading numbers in the laboratory. It also makes it very difficult for the laboratory to ensure all samples are present or to check on missing or broken samples.

DO NOT label containers with water- soluble ink. It smudges when wet and may rub off if samples are chilled or frozen.

Serum can be frozen, provided there are no blood cells present in it

IV. Haemolysed samples

Haemolysis can occur as a result of poor collection technique, contaminated equipment or poor handling of the sample once it is collected. Common causes of haemolysis include:

- Use of non-sterile containers for collection or storage.
- Contamination by faecal and other material due to faulty aseptic techniques.
- Contaminating of the sample by water.
- A slow flow from the needle, due to obstruction of the needle, or failure to insert into mid-vein.
- Forcibly expelling blood through a needle.
- Heating of samples, usually in car boots or through back windows or car, or after prolonged exposure to direct sun light during collection.
- Freezing.

Note: *Pig blood haemolyses quickly. Serum should always be separated from the clot within four hours of collection.*

V. Storage of specimens prior to dispatch

Samples should be allowed to clot before transporting them over any distance. Clots may not retract readily in cold weather or if they are chilled too soon after collection. Sample should be held in a warm room until the clot is retracted. Once the clot has retracted, blood samples must be held chilled to reduce contamination, haemolysis and autolysis.

If the laboratory will not get the samples within 48 hours of collection, decant the serum into a 5ml sterile, screw-capped plastic container and submit the serum sample only. If virological examination of the clot is also needed (Pestivirus antigen detection), submit clot separately.

F. Waste disposal

- Decontaminate instruments before cleaning them.
- Clean and disinfect all work surfaces or the premises where necropsy was conducted.
- Decontaminate self (e.g., disinfect and remove boots, gloves, and coveralls).

G. Risk assessment

- Protective clothing, including boots, gloves, face mask and goggles must be worn. The carcasses should be burned or deep buried under stones with quick-lime.
- All carcasses should be handled with care especially
- No eating, drinking, grooming, or other activities that are a means of exposure are permitted in necropsy areas.
- Transport unfixed tissues in leak-proof containers.

12.6 Annexure 6: Standard operating procedure for Rose Bengal Test (RBT) for brucella

A. Introduction

Brucella is a small Gram-negative bacterium (0.4-0.8 µm in diameter and 0.4-3.0 µm in length) which is non-flagellated, and non-spore forming. Four species are pathogenic to human: *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*. All four species are exciters of Brucellosis, a disease characterized by undulating fever. Depending on exciter the disease is also called Morbus Bang (*B. abortus*) or Malta fever (*B. melitensis*).The pathogens are transmitted from animals, which are mainly affected. The infection is caused by contact with ill animals or their excrements as well as by non-pasteurized milk and milk products like fresh cheese from sheep or goat. Main entrances are skin wounds, conjunctives and digestive tract. The intact pathogens are transported by granulocytes into local lymph nodes, from where they spread haematogenous. All kind of organs can be infected.

B. Principles

The Rose Bengal test is a simple agglutination test in which a standardized antigen with added Rose Bengal dye is mixed with serum on a standardized, clear glass or plastic substrate. After standardised mixing for a standard time at a standard temperature, the degree of agglutination is estimated over a light box in comparison with positive controls.

The Rose Bengal test is relatively simple to perform and interpret and can be done by laboratories with basic resources.

C. Application

The Rose Bengal test is a spot agglutination test used to screen herds for *Brucella abortus*. For the diagnosis of individual cattle the test is oversensitive. This test is also used to test for *B. suis*.

D. Objective

To describe the procedures for screening the herd against Brucellosis

E. Apparatus/test kit/reagents

- *B. abortus* Rose Bengal antigen.
- *B. abortus* positive reference control serum
- *B. abortus* negative reference control serum
- Agglutination plates, flat, Perspex
- Pipettors and tips
- Spatula for mixing serum and antigen
- Timer

F. Test procedure

- Bring antigen and test sera to room temperature ($20^0C \pm 5^0C$).
- Pipette 25-30 µl of test serum, and positive and negative reference control serum onto a labelled agglutination plate.
- Add 25-30 µl of antigen to each well. One plate at a time is set up to minimise delay between the addition of antigen to the first and last serum.
- Mix antigen and sera with a mixer / spatula, wiping the implement between each mix.
- Place the agglutination plate on a tray rocker (approximately 30 oscillations / minute) and mix for 4 min.
- Read the results using a light box.
- Read results of reference sera first, then the remaining wells.

G. Result interpretation and reporting

A scoring system is used to allow correlation with the CFT results. The following allows distinction of degrees of reaction:

Negative	No agglutination, no "ringing", a uniform pink color.
1+	Barely perceptible agglutination and/or "ringing". Also any doubtful reaction.
2+	Fine agglutination, definite ringing, and some clearing.
3+	Coarse clumping, definite clearing.

Results are recorded as Negative or Positive in standard laboratory report format.

H. Waste disposal

The samples and the kits items contain infectious materials hence, should be disposed properly.

I. Risk assessment

All Positive and Suspect samples are retested to confirm results using *B. abortus* ELISA test. It can infect the handlers if adequate precautions are not taken.

12.7 Annexure 7: Standard operating procedure for ELISA for brucella abortus

A. Introduction

Brucella is a small Gram-negative bacterium (0.4-0.8 µm in diameter and 0.4-3.0 µm in length) which is non-flagellated, and non-spore forming. Four species are pathogenic to human: *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*. All four species are exciters of Brucellosis, a disease characterized by undulating fever. Depending on exciter the disease is also called Morbus Bang (*B. abortus*) or Malta fever (*B. melitensis*).

The pathogens are transmitted from animals, which are mainly affected. The infection is caused by contact with ill animals or their excrements as well as by non-pasteurized milk and milk products like fresh cheese from sheep or goat. Main entrances are skin wounds, conjunctives and digestive tract. The intact pathogens are transported by granulocytes into local lymph nodes, from where they spread haematogenous. All kind of organs can be infected.

B. Principles

Microtiter plates are coated with inactivated antigen. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for *B. abortus* binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidise-labelled ani-ruminant IgG Conjugate is added, which binds to the ruminant antibody complex with the *B. abortus* antigen. Unbound conjugate is removed by washing and TMB substrate added to the wells. The degree of color that develops (Optical density) measured at 450nm) is directly proportional to the amount of antibody specific for *B. abortus* present in the sample. The result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the Positive Control.

C. Application

The IDEXX Brucellosis Serum X2 test kit provides a rapid, simple, sensitive and specific method for detecting antibodies against *Brucella abortus* in individual serum and in pools of up to 10 individual serum samples from ruminants.

D. Objective

To describe the procedure for detecting antibodies against *Brucellaabortus* inserum samples from ruminants.

E. Apparatus/test kit/reagents

- IDEXX Brucellosis Serum X2
- *B. abortus* antigen coated plate
- Positive control
- Negative control
- Conjugate
- TMB SubstrateN12
- Stop Solution N.3
- Wash Concentration (10X). Dilute the wash Concentrate (10X) 1:10 with distilled water.

F. Test procedure

- All the reagents must be allowed to come to 18-26 °C before use. Mix reagents by gently inverting or swirling.
- Obtain coated plate and record the sample position
- Dispense 90µL wash solution into each well
- Dispense 10 µL of the un diluted positive control into duplicate wells
- Dispense 10 µL of the un diluted Negative control into duplicate wells
- Dispense 10 µL of the un diluted serum samples or pools of up to 10 serum samples in to appropriate wells
- Mix the content of the micro wells by gently tapping the plate or use micro plate shaker.
- Short Incubation-Cover the plate and incubate for 60 minutes at 37^{o}C. The plate should be tightly sealed or incubated in a humid chamber using plate covers.
- Overnight incubation- Cover the plate and incubate overnight for 14-18 hours at 2-8^{o}C. The plate should be tightly sealed or incubated in a humid chamber using plate covers
- Remove the solution and wash each well with 300 µL of wash solution 3 times. Avoid the plate dying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- Dispense 100 µL conjugate in to each well.
- Incubate for 60 minutes at 37oC. The plate should be tightly sealed or incubate in a humid chamber using plate covers.
- Repeat step 9
- Dispense 100 µL of TMB substrate N.12 into each well.
- Incubate at 18-26oC for 15 minutes.
- Dispense 100 µL of stop solution N.3 into each well.
- Read the results at a wavelength of 450 nm.

G. Result interpretation

$$SP\% = 100 * (sample\ A\ (450) - NC)/(PC - NC)$$

Negative - SP % < 80

Positive - SP % ≥ 80

H. Waste disposal

Treat it as the infectious material and dispose accordingly.

I. Risk assessment

- Handle all reagents and samples as bio-hazardous material.
- Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontami-nated with bleach or other strong oxidizing agent before disposal
- Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be com- promised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
- The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
- Do not use this kit after the expiration date.
- Never pipette by mouth. Allow all reagents to come to room temperature before starting.

12.8 Annexure 8: Standard operating procedure for specimen collection for isolation of brucella organism from infected and culled animals (cattle)

A. Introduction

Culture and isolation of Brucella organism is one of the confirmatory tests in the screening process for Brucellosis control and eradication programme. Brucellosis in cattle is predominantly caused by B abortus. Different biovars of B abortus are involved in infection. Collection of appropriate tissue samples is very important for recovery of organism in laboratory settings. Recovered organism can be genetically characterized to determine the molecular epidemiology of B abortus.

B. Principles

Collection of quality sample is important for successful recovery of organism; confirm diagnosis and use in down-stream processing

C. Purpose

To outline the proper procedures for performing a necropsy and post mortem examination in livestock. Necropsy is to be performed for diagnostic purpose, disease outbreak and research.

D. Application/scope

This SOP is intended for individual involved in test and cull method of Brucellosis control and eradication programme. This SOP is also useful for cattle farm workers who are involved in daily management of animals including calving and abortion.

E. Objective

This SOP serves as guide to field animal health workers (veterinary professional, veterinary paraprofessionals, farm attendants and test and cull team members) for collection of appropriate specimens.

F. Apparatus/equipment

- Rubber boots, protective clothing, rubber apron or lab coat, rubber gloves.
- Knives – large and small, with sharpening steel.
- Scissors (various sizes) and saws; bone cutters
- Number of sterile, open-mouthed screw top jars of different sizes for specimen
- Sterile swabs in test tubes
- Spatula
- Plastic bags for specimens

- Petri dishes
- Labels
- Soap, water, disinfectant and towel.

G. Reagents, solution and buffer

- PBS
- 70% alcohol for wetting and disinfecting the skin,
- 96-100% ethyl alcohol (for fixing specimens for PCR testing
- Normal saline (0.85% NaCl) with a pipette (for parasitological examination),

H. Procedure

I. Preparation

- Animal necropsy should be performed in areas specifically designated for that purpose in the laboratories or identify an ideal location in the farms or the areas
- Proper personal protective equipment and attire must be worn when performing necropsy
- Disposable gloves, shoe covers, and gown must be worn when conducting a necropsy
- Full protective clothing must be worn when handling animals infected with biohazardous materials or chemical carcinogens and includes double gloves, cap, disposable gown, shoe covers, mask (or respirator if required), and eye protection
- Obtain and record the animal and/or herd history
- Make sure appropriate disinfectant is available
- Carefully note external abnormalities, check orifices, genitals and mammary glands
- Make note of any injuries, wounds, parasites
- Observe the general appearance of carcass: rigor mortis, nasal and anal tissues, wounds, enlargements, eyes, skin lesion, condition of flesh and visible mucous membranes.

II. Necropsy procedure

- Cattle are best positioned on their left side
- Make an external examination and place ectoparasites in 70% ethanol. Conduct a thorough external examination by reviewing the body surface and orifices for abnormalities. Palpate for superficial swellings, or for enlarged organs or masses within body cavities
- To prevent contamination, disinfect the skin or use clean instruments to open body cavities. Open the abdominal and thoracic cavities carefully so as to prevent contamination from the outside or from a cut organ
- Observe, but do not disturb, organ placement, noting any abnormalities. Examine organs and tissues *in situ* before dissecting or collecting tissues and record any abnormalities
- With a syringe, aseptically collect a specimen of any abnormal body fluid
- Aseptically collect specimens of liver, kidney, spleen, and lymph node
- Aseptically collect specimens of lung and heart

- Indicate on the necropsy form which tissues are collected or sampled that are to be submitted for other tests
- All lesions that were observed during the examination, or that are observed during the necropsy must be recorded on the appropriate necropsy form, and include a complete description (e.g., size, number, color, shape, texture, severity, and weight or volume as appropriate) as far as possible.

I. Waste disposal

- Decontaminate instruments before cleaning them.
- Clean and disinfect all work surfaces or the premises where necropsy was conducted.
- Decontaminate self (e.g., disinfect and remove boots, gloves, and coveralls).

J. Risk assessment

- Protective clothing, including boots, gloves, face mask and goggles must be worn. The carcasses should be burned or deep buried under stones with quick-lime.
- All carcasses should be handled with care especially
- No eating, drinking, grooming, or other activities that are a means of exposure are permitted in necropsy areas.
- Transport unfixed tissues in leak-proof containers.

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