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**Molecular Diagnosis of *Brucella* *abortus* in cattle**

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Roll No: 0213/02

Registration No: 157

Session: 2013-2014

**A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Medicine**

**Department of Medicine & Surgury**

**Faculty of Veterinary Medicine**

**Chittagong Veterinary and Animal Sciences University**

**Chittagong-4225, Bangladesh**

**December 2014**

***Dedication***

*I dedicate this small piece of work*

*to my beloved mother*

*who always valued education*

*above everything else*

# 

# Authorization

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**This is to certify that we have examined the above Master’s thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made**

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**December 2014**

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*The Author*

# Abbreviations

|  |  |
| --- | --- |
| AGID : Agar Gel Immunodiffusion | IFA : Indirect Fluorescent Antibody |
| AI : Artificial Insemination | LPS : Lipo-polysaccharide |
| BAPAT : Buffer Acidified Plate Test | MRT : Milk Ring Test |
| BAU : Bangladesh Agricultural University | NS : Natural Service |
| BCS : Body Condition Scoring | OD : Optical Density |
| BPA : Buffered Plate Antigen | OIE : World Organization for Animal Health |
| BPAT : Buffered Antigen Plate Agglutination Test | OMP : Outer Membrane Protein |
| BQ : Black Quarter | OPS : Oligo-polysaccharide |
| CI : Confidence Interval | *P* : probability |
| CPI : Credible Posterior Interval | PCR : Polymerase Chain Reaction |
| CVASU : Chittagong Veterinary and Animal Sciences University | PRTC : Poultry Research and Training Center |
| DANIDA : Danish International Development Agency | R : Rough |
| DMS : Department of Medicine and Surgery | RBPT / RBT: Rose Bengal Plate Test |
| ELISA : Enzyme Linked Immunosorbent Assay | S : Smooth |
| FAO : Food and Agricultural Organization | S19 : Strain 19 |
| FMD : Food and Mouth Disease | SAT : Slow Agglutination Test |
| FPA : Fluorescence Polarization Assay | Se : Sensitivity |
| FVM : Faculty of Veterinary Medicine | Sp : Specificity |
| GDP : Gross Domestic Product | TAT / AT: Tube Agglutination Test |
| HPRO : Horse Reddish Per-oxidase | USDA: United States Department of Agriculture |
| HS : Haemorrhagic Septicemia | WHO : World Health Organization |

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# Abstract

One of the world’s most important zoonotic diseases like brucellosis, affected both animals and human robustly. In Bangladesh, late abortion commonly explored in dairy cattle by diverse types of causal agents where *Brucella abortus* is one of the major causal agents. In the study, this organism was isolated and identified through molecular characterization from aborted fetus of dairy cattle first time ever in Bangladesh. For the study, 25 aborted fetuses were collected and tested from 25 sero-positive dairy farm of Chittagong district. A total of 20% samples were found positive as *Brucella abortus* through culture and conventional PCR technique which is an authentication for the infectious agent. The organism varies from organ to organ likely eyeball (20%), liver (16%), lung (12%) and kidney (08%) according to sensitive and specific location. These findings were considered to be more useful in prevention and controlling against Brucellosis at Chittagong as well as in Bangladesh.

**Keywords**: *Brucella abortus*, Cattle, aborted fetus, PCR, characterization.

# CHAPTER 1

# INTRODUCTION

In Bangladesh, approximately 80 percent of people live in villages, and rural income is largely dependend on livestock; the people are close contact with livestock on a daily life. There is little information available about the prevalence of brucellosis in human beings and animals in the country (Rahman et al., 1983; Mustafa, 1984). Brucellosis is one of the world’s most major zoonoses, alongside tuberculosis and rabies (Boschiroli et al., 2001). Brucellosis is an important disease caused by gram – negative bacteria *Brucella* that are pathogenic for a wide variety of animals and humans (Sommerville et al., 2003). The principal manifestations of bovine brucellosis are reproductive failure i.e. abortion in cows; orchitis and epididymitis in bulls. Brucellosis in human beings is usually characterized by influenza – like clinical disease, which may be severe and may be followed by chronic intermittent relapses (Hugh – Jones, 2000). The genus Brucella has six recognized species on the basis of host specificity but the greatest economic impact results from *Brucella abortus*. The prevalence of brucellosis was 15% by the Rose Bengal Test (RBT) and Plate Agglutination Test (PAT), and 10% by the Tube Agglutination Test (TAT) in cows that had a previous abortion (Rahman et al., 2006). In cows with a history of retained placenta, the prevalence of brucellosis was 13·04% by the RBT and PAT, and 8·70% by the TAT. The prevalence of brucellosis in repeat breeding cases was 1·45% by all three serological tests. There was a statistically significant difference in the prevalence of the disease between cows with a history of retained placenta and repeat breeding cases (P<0·05). The prevalence of brucellosis in cows that had mastitis was 4·76% by the RBT and PAT, but was not demonstrated by the TAT. The prevalence of brucellosis in cows in Bangladesh is not negligible, and it is therefore worth considering the adoption of preventive measures (Rahman et al., 2006).

Dairy cattle industry is one of the major sub-sectors of animal agriculture (Livestock) in Bangladesh. Gestation is a biological phenomenon of all mammalian species including dairy cattle (Noakes et al., 2009). Early and late termination of gestation of dairy cows is called abortion (Noakes et al., 2009). Many etiologies of abortion also cause stillbirths, mummification, and weak or deformed neonates. The etiologic diagnosis of abortion in livestock is a difficult and often frustrating task. The diagnostic success rate is relatively low: 30 – 40 % for bovine, 60 – 65% for ovine, and 35 – 40% for porcine of abortion cases submitted to diagnostic laboratories (Cooper, 2012). Abortion causes the loss of calf crop as well as milk production of animal. As a result, abortion in dairy animal is a great threat of dairy industry all over the world. It hinders the milk and calf crop production and ultimately loosing concern of farming community all over the globe.

A cross sectional survey was conducted to determine the seroprevalence of brucellosis in cattle in Bangladesh Agricultural University (BAU) Veterinary Clinics, in BAU Dairy Farm and Vabokhali from June 2008 to November 2008 (Rahman et al., 2009). A total of 200 serum samples were collected from BAU Veterinary Clinic, from BAU Dairy Farm and Vabokhali. Among the serum samples 143 sera samples were collected from BAU Veterinary Clinic, 42 serum samples from BAU Dairy Farm and 15 serum samples from Vabokhali. Sera were separated from blood samples and tested with specific *Brucella abortus* antigen (BAA) test and *B. melitensis* antigen (BMA) test. The overall seroprevalence of brucellosis in cattle was 5% in BAA and 0.5% in BMA (Rahman et al., 2009).

The importance of brucellosis is not known precisely, but it can have a considerable impact on both human and animal health, as well as having socioeconomic effects, especially in areas where rural income relies largely on livestock breeding and dairy products. In animals, brucellosis mainly affects reproduction and fertility, reduces the survival of newborns and reduces milk yield (Roth et al., 2003). There are six species of *Brucella,* recognized according to host specificity; *Brucella abortus* is responsible for infection in cattle (Bricker and Halling, 1994). In 2005, a studied the prevalence of *Brucella* antibodies in sera of cows in Bangladesh (Amin et al.,2005).

The organism causes late abortion in cattle and cause economic impact on animal (approximately 60,000 BDT / Cow / Year). Therefore, the present study is designed to investigate the prevalence of late abortion of bovine that caused by *Brucella* subsequently isolation, identification and characterization of them for selecting the vaccine candidature.

**Objectives:**

* To isolate and identify the *Brucella abortus* from fetal samples of dairy cows.
* Estimation of prevalence of bovine brucellosis
* To characterize the isolated *Brucella abortus* at molecular level through PCR .

# CHAPTER 2

# REVIEW OF LITERATURE

## The observation and findings of various authors on the isolation, growth characteristics, and identification of *Brucella abortus* bacteria pertinent to this study are presented as follows:

### 2.1. Etiology of Brucellosis

In humans, brucellosis can be caused by *B. abortus*, *B. melitensis*, *B. suis* biovars 1-4 and, rarely, *B. canis* or marine mammal *Brucella* (Acha and Szyfres, 2003; Brew et al*.,* 1999 and McDonald *et al.,* 2006). *B. abortus* and *B. suis* infections usually affect occupational groups, while *B. melitensis* infections occur more frequently than the other *Brucella* species in the general population (Acha and Szyfres, 2003; De Massis et al., 2005). Consumption of sheep or goat milk containing *B. melitensis* is an important source of human brucellosis worldwide and has caused several outbreaks. For example; in some countries including Italy, 99% of human brucellosis is caused by *B. melitensis* (De Massis et al., 2005)

Brucellosis results from infection by various species of *Brucella*, a Gram negative, facultative intracellular coccobacillus or short rod in the family Brucellaceae. Six named species occur in animals: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Foster et al., 2007). One or more unnamed species of *Brucella* have been found in marine mammals. Formal names proposed for marine mammal isolates are *B. maris* for all strains, or *B. pinnipediae* for strains from pinnipeds (seals, sea lions and walruses) and *B. cetaceae* for isolates from cetaceans (whales, porpoises and dolphins) (Foster *et al.,* 2007). Some species of *Brucella* contain biovars. Seven biovars are recognized for *B. abortus* (1,7-10,12,13), three for *B. melitensis* (1, 7,8), and five for *B. suis*. The other species have not been differentiated into biovars, although variants exist (Corbel and Thomas, 1985).

Each *Brucella* species is associated most often with certain hosts. *B. abortus* usually causes brucellosis in cattle, bison and buffalo (Acha and Szyfres, 2003), *B. melitensis* is the most important species in sheep and goats, but *B. ovis* can also cause infertility in rams. *B. canis* causes disease almost exclusively in dogs. It can cause disease in humans, although this is rare even in countries where the infection is common in dogs (Carmichael, 1990).

## 2.2. Geographic Distribution

Brucellosis is found worldwide but it is well controlled in most developed countries (Corbel, 1997). Clinical disease is still common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the Caribbean. *Brucella* species vary in their geographic distribution. *B. abortus* is found worldwide in cattle-raising regions except in Japan, Canada, some European countries, Australia, New Zealand and Israel, where it has been eradicated (Corbel, 1997). Eradication from domesticated herds is nearly complete in the U.S. *B. abortus* persists in wildlife hosts in some regions, including the Greater Yellowstone Area of North America. *B. melitensis* is particularly common in the Mediterranean. It also occurs in the Middle East and Central Asia, around the Arabian Gulf and in some countries of Central America. This organism has been reported from Africa and India, but it does not seem to be endemic in northern Europe, North America (except Mexico), Southeast Asia, Australia or New Zealand (Amato, 1995). *B. ovis* probably occurs in most sheep-raising regions of the world. It has been reported from Australia, New Zealand, North and South America, South Africa and many countries in Europe (Ashenafi et al*.,* 2007).

In the past, *B. suis* was found worldwide in swine-raising regions. This organism has been eradicated from domesticated pigs in the U.S., Canada, many European countries and other nations. However, it persists in wild and/ or feral swine populations in some areas, including the U.S., Europe and Queensland, Australia (Godfroid & Kasbohrer, 2002). Sporadic outbreaks are reported in domesticated herds or humans due to transmission from this source. *B. suis* continues to occur in domesticated herds in some countries of South and Central America (including Mexico) and Asia. *B. suis* biovars-1 and 3 are found worldwide, but other biovars have a limited geographic distribution. Biovar-2 occurs in wild boar in much of Europe (Godfroid & Kasbohrer, 2002). Biovar-4 (rangiferine brucellosis) is limited to the Arctic regions of North America and Russia including Siberia, Canada and Alaska (Lorry, 1991). Biovar-5 (murine brucellosis) is found in the former USSR.

## 2.3. Transmission

Common sources of infection for people include contact with animal abortion products; ingestion of unpasteurized dairy products from cows (Acha and Szyfres, 2003), *B. abortus, B. melitensis*, *B. suis* and *B. canis* are usually transmitted between animals by contact with the placenta, fetus, fetal fluids and vaginal discharges from an infected animal. Animals are infectious after either an abortion or full term parturition. Although ruminants are usually asymptomatic after their first abortion, they can become chronic carriers, and continue to shed *Brucella* in milk and uterine discharges during subsequent pregnancies. Dogs may also shed *B. canis* in later pregnancies, with or without symptoms. Entry into the body occurs by ingestion and through the mucous membranes, broken skin and possibly intact skin (De Massis et al., 2005).

*Brucella* can be spread on fomites including feed and water. In conditions of high humidity, low temperatures, and no sunlight, these organisms can remain viable for several months in water, aborted fetuses, manure, wool, hay, equipment and clothes. *Brucella* can withstand drying, particularly when organic material is present, and can survive in dust and soil. Survival is longer when the temperature is low, particularly when it is below freezing.

Brucellosis in human beings is caused by exposure to livestock and livestock products. Infection can result from direct contact with infected animals and can also be transmitted to consumers through raw milk and milk products. Brucellosis spreads between animals in a herd and the disease is a systemic infection that can involve many organs and tissues. Once the acute period of the disease is over, symptoms of brucellosis are mostly not pathognomonic, and the organism can be chronically located in the supramammary lymphatic nodes and mammary glands of 80% of infected animals. Thus they continue to secrete the *Brucella* organism in their body fluids (Redkar et al., 2001).

## 2.4. Disinfection

*Brucella* species are readily killed by most commonly available disinfectants including hypochlorite solutions, 70% ethanol, isopropanol, iodophores, phenolic disinfectants, formaldehyde, glutaraldehyde and xylene; however, organic matter and low temperatures decrease the efficacy of disinfectants. Disinfectants reported to destroy *Brucella* on contaminated surfaces include 2.5% sodium hypochlorite, 2-3% caustic soda, 20% freshly slaked lime suspension, or 2% formaldehyde solution (all tested for one hour). Ethanol, isopropanol, iodophores, substituted phenols or diluted hypochlorite solutions can be used on contaminated skin (Radostits et al., 2000). The fermentation time necessary to ensure safety in ripened, fermented cheeses in unknown, but is estimated to be approximately three months. *Brucella* is reported to persist for weeks in ice cream and months in butter. This organism survives for very short periods in meat, unless it is frozen; in frozen meat, survival times of years have been reported (Radostits et al., 2000).

### 2.5. Species Affected

Most species of *Brucella* are maintained in a limited number of reservoir hosts. Maintenance hosts for *Brucella abortus* include cattle (Radostits et al., 2000), bison (*Bison* spp.) water buffalo (*Bubalus bubalus*), African buffalo (*Syncerus caffer*), elk and camels (Moreno et al., 2002). A feral pig population was recently reported to maintain *B. abortus* in the U.S. Sheep and goats are the reservoir hosts for *B. melitensis*. Sheep are also the maintenance hosts for *B. ovis*. In addition, *B. ovis* occurs in farmed red deer (*Odocoileus virginianus*) in New Zealand. *B. canis* is maintained in dogs and *B. neotomae* in rodents. *B. suis* contains more diverse isolates than other *Brucella* species, and these isolates have broader host specificity. *B. suis* biovars-1, 2 and 3 affect swine (Jacques *et al.,* 2007). Biovars-1 and 3 are found in both domesticated pigs (*Sus scrofa domesticus*) and wild or feral pigs (Corbel et al., 1920; Moreno et al., 2002). Biovar-2 currently occurs mainly in wild boar (*Sus scrofa scrofa*) and European hares (*Lepus capensis*); however, this biovar can be transmitted from these reservoirs to domesticated pigs, and spreads readily in these herds. Biovar-4 is maintained in caribou and reindeer (*Rangifer tarandus* and its various subspecies). Biovar-5 is found in small rodents. Marine *Brucella* species have been found by culture or serology in many pinniped and cetacean species including seals, sea lions, walruses, porpoises, dolphins, whales and a European otter (Foster et al., 2007).

## 2.6. Incubation Period

The incubation period varies with the species and stage of gestation at infection. In cattle, reproductive losses typically occur during the second half of the pregnancy; thus, the incubation period is longer when animals are infected early in gestation. In this species, abortions and stillbirths usually occur two weeks to five months after infection. In pigs, abortions can occur at any time during gestation. In dogs, abortions are most common at approximately 7 to 9 weeks of gestation, but early embryonic deaths have also been reported after 2 to 3 weeks (Acha and Szyfres, 2003).

**2.5.1. Clinical signs**

2.5.2. Bovine brucellosis (B. abortus***)***

In cattle, *B. abortus* causes abortion, stillbirth and weak calve; abortions usually occur during the second half of gestation, the placenta may be retained and lactation may be decreased (Acha and Szyfres, 2003). After the first abortion, subsequent pregnancies are generally normal; however, cows may shed the organism in milk and uterine discharges. Epididymitis, seminal vesiculitis, orchitis and testicular abscesses are sometimes seen in bulls. Infertility occurs occasionally in both sexes, due to metritis or orchitis/epididymitis (Acha and Szyfres, 2003). Hygromas, particularly on the leg joints, are a common symptom in some tropical countries. Arthritis can develop after long-term infections. Systemic signs do not usually occur in uncomplicated infections, and deaths are rare except in the fetus or newborn. Infections in nonpregnant females are usually asymptomatic. The brucellae localize in the supra-mammary lymph nodes and mammary glands of 80% of the infected animals and thus continue to secrete the pathogen in milk throughout their lives (Hamdy and Amin, 2002). Most infected cows abort only once although the placenta will be heavily infected at subsequent apparently normal calvings (Morgan, 1969).

Camels can be infected by *B. abortus* and *B. melitensis* when they are pastured together with infected sheep, goats and cattle. Milk from infected camels represents a major source of infection that is underestimated in the Middle East (Musa et al., 2008).

## 2.5.3. Ovine and caprine brucellosis (B. melitensis)

*B. melitensis* mainly causes abortions, stillbirths and the birth of weak offspring (Lilenbaum et al., 2007). Animals that abort may retain the placenta. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies Milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal birth. However, clinical signs of mastitis are uncommon. Acute orchitis and epididymitis can occur in males, and may result in infertility. Arthritis is seen occasionally in both sexes. Many non-pregnant sheep and goats remain asymptomatic (Acha and Szyfres, 2003).

## 2.5.4. Ovine epididymitis (B. ovis)

*B. ovis* affects sheep but not goats. This organism can cause epididymitis, orchitis and impaired fertility in rams (Acha and Szyfres, 2003). Initially, only poor quality semen may be seen; later, lesions may be palpable in the epididymis and scrotum. Epididymitis may be unilateral or, occasionally, bilateral. The testes may atrophy. Some rams shed *B. ovis* for long periods without clinically apparent lesions. Abortions, placentitis and perinatal mortality can be seen in ewes but are uncommon. Systemic signs are rare. *B. ovis* can also cause poor semen quality in red deer stags, but abortions have not been reported in hinds.

## 2.5.5 Porcine and rangiferine brucellosis (B. suis)

In pigs, the most common symptoms are abortion, which can occur at any time during gestation, and weak or stillborn piglets. Vaginal discharge is often minimal and abortions may be mistaken for infertility. Occasionally, some sows develop metritis. Temporary or permanent orchitis can be seen in boars (Radostits et al., 2000). Boars can also shed *B. suis* asymptomatically in the semen; sterility may be the only sign of infection. Swollen joints and tendon sheaths, accompanied by lameness and incoordination, can occur in both sexes. Less common signs include posterior paralysis, spondylitis and abscesses in various organs (Radostits et al., 2000). Although some pigs recover, others remain permanently infected. Fertility can be permanently impaired, particularly in boars. Some animals remain asymptomatic.

## 2.5.6. Canine brucellosis (B. canis)

*B. canis* can cause abortions and stillbirths in pregnant dogs (Acha and Szyfres, 2003). Most abortions occur late, particularly during the seventh to ninth week of gestation. Abortions are usually followed by a mucoid, serosanguinous or gray-green vaginal discharge that persists for up to six weeks. Early embryonic deaths and resorption have been reported a few weeks after mating, and may be mistaken for failure to conceive. Some pups are born live but weak; most die soon after birth. Other congenitally infected pups can be born normal and later develop brucellosis. Clinical signs occur during subsequent pregnancies in some dogs, but not in others. Epididymitis, scrotal edema, orchitis and poor sperm quality may be seen in males. Scrotal dermatitis can occur due to self-trauma. Unilateral or bilateral testicular atrophy can be seen in chronic infections, and some males become infertile.

Lymphadenitis is common in infected dogs. Lethargy or fatigue, exercise intolerance, decreased appetite, weight loss and behavioral abnormalities (loss of alertness, poor performance of tasks) are occasionally reported; however, most affected dogs do not appear seriously ill. Occasionally, discospondylitis of the thoracic and/or lumbar vertebrae can cause stiffness, lameness or back pain. Uveitis, endophthalmitis, polygranulomatous dermatitis, endocarditis and meningoencephalitis have also been reported (Acha and Szyfres, 2003). Fever is uncommon, and deaths are rare except in the fetus or newborn. Many infected dogs remain asymptomatic.

## 2.5.7. Brucellosis in horses

In horses, *B. abortus* and occasionally *B. suis* can cause inflammation of the supraspinous or supra-atlantal bursa; these syndromes are known, respectively, as fistulous withers or poll evil. The bursal sac becomes distended by a clear, viscous, straw-colored exudate and develops a thickened wall. It can rupture, leading to secondary inflammation. In chronic cases, nearby ligaments and the dorsal vertebral spines may become necrotic (Jacques et al., 2007). *Brucella*-associated abortions are rare in horses.

## 2.5.8 Brucellosis in marine mammals

There is little information on the effects of brucellosis in marine mammals. Reproductive disease is difficult to assess in wild animals, but *Brucella* has been isolated from the reproductive organs of some marine species. In rare cases, infections have also been linked to lesions or clinical disease. *Brucella*-associated abortions and placentitis were reported in two captive bottlenose dolphins. Lesions consistent with a possible abortion were also reported in a wild Atlantic white-sided dolphin. Recently, *Brucella* was isolated from a dead newborn Maui’s dolphin in New Zealand; the animal was born alive but died before taking its first breath. *Brucella*-associated epididymitis has been reported in porpoises, and orchitis from suspected brucellosis was reported in minke whales (Foster et al., 2007).

*Brucella* infections have been linked with systemic disease in a few marine mammals. *Brucella*-associated meningoencephalitis was reported in three stranded striped dolphins. Other signs of *Brucella*-associated systemic disease have been seen mainly in Atlantic white-sided dolphins; the lesions included hepatic and splenic necrosis, lymphadenitis and mastitis. *Brucella* has also been identified as a possible secondary invader or opportunistic pathogen in debilitated seals, dolphins and porpoises (Scholz et al., 2008). It has been isolated from several subcutaneous abscesses. In addition, this organism has been found in organs with no microscopic or gross lesions, and in apparently healthy animals.

## 2.6. Diagnostic of brucellosis

Brucellosis can be diagnosed by culture, serology, PCR or other tests.

## 2.6.1. Microscopic examination

Microscopic examination of smears stained with the Stamp's modification of the Ziehl-Neelsen method can be used for a presumptive diagnosis (Joshi et al., 2005). Organisms may be found in aborted products, vaginal discharges, milk, semen or various tissues. *Brucell*a species are not truely acid-fast, but they are resistant to decolorization by weak acids, and stain red against a blue background. Brucellae are coccobacilli or short rods, usually arranged singly but sometimes in pairs or small groups. This test is not definitive. Other organisms such as *Chlamydophila abortus* and *Coxiella burnetii* can resemble *Brucella*. Direct examination may not detect the small numbers of organisms present in milk and dairy products. Because of the slowness of the culture one often moves to serology (Al Dahouk et al., 2003).

### 2.6.2. Culture in medium

Culture from the blood of a patient provides definite proof of brucellosis (Al Dahouk etal., 2003). *Brucella* species can be recovered from numerous tissues and secretions, particularly fetal membranes, vaginal secretions, milk (or udder secretions in nonlactating cow), semen, arthritis or hygroma fluids, and the stomach contents, spleen and lung from aborted fetuses (Radostits et al., 2000). Blood cultures are often used to detect *B. canis* in dogs. In this species, bacteremia (which may be intermittent) can persist for up to five years and possibly longer. Oral, nasal, tracheal, vaginal and anal swabs, as well as feces, can be submitted for culture from marine mammals (Radostits et al., 2000).

At necropsy, bacteria can be isolated from a variety of organs including lymph nodes, spleen, uterus, udder, testis, epididymis, joint exudate, abscesses and other affected tissues. In ruminants with suspected *B. abortus* or *B. melitensis* infections, the spleen, mammary and genital lymph nodes, udder and late pregnant or early post-parturient uterus are the most reliable samples to collect. The preferred tissues to collect in rams suspected of *B. ovis* infection are the epididymis, seminal vesicles, ampullae and inguinal lymph nodes. In dogs, recommended biopsy or necropsy samples include lymph nodes, prostate, epididymis, testis, uterus, spleen, liver and bone marrow (Radostits et al., 2000). The lymph nodes and spleen are most likely to be positive in non-bacteremic dogs.

*Brucella* spp. can be isolated on a variety of plain media, or selective media such as Farrell's medium or Thayer-Martin’s modified medium. Enrichment techniques can also be used. Colony morphology varies with the species. Colonies of smooth forms (*B. abortus*, *B. suis, B. melitensis* and marine mammal *Brucella*) are round with smooth margins (Poester et al., 2010).

Animal inoculation is rarely used to isolate *Brucella*, but may be necessary if other techniques fail. Guinea pigs or mice can be used.

## 2.6.3. Serology

Brucellosis is often diagnosed by serology. Serological tests are not completely specific and cannot always distinguish reactions because most serological tests rely on the unique antigenic properties of lipopolysaccharides (LPS) that are shared among the *Brucella* species and the use of LPS as antigen causes cross-reactivity with organisms such as *Vibrio* and *Yersinia enterocolitica* that share common features of the LPS (Munoz et al., 2005).

In cattle, sheep and goats, serology can be used for a presumptive diagnosis of brucellosis, or to screen herds. Serological tests commonly used to test individual cattle or herds include the buffered *Brucella* antigen tests (rose Bengal test and buffered plate agglutination test), complement fixation, indirect or competitive enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarization assay. The classical Rose Bengal test (RB) is often used as a rapid screening test (Ruiz-Mesa et al., 2005). For confirmation of RB the Wright or serum agglutination test (SAT) or in more sophisticated equipped laboratories enzyme linked immunosorbent assay (ELISA) may be used (Munoz et al., 2005). Rivanol precipitation, acidified antigen procedures and the serum agglutination test (tube or microtiter test) are also available. Supplemental tests such as complement fixation or rivanol precipitation are often used to clarify the results from plate or card agglutination tests. ELISAs or the *Brucella* milk ring test (BRT) can be used to screen herds by detecting antibodies in milk. In USA, the two primary methods of testing of brucellosis in cattle are: the Brucella ring test (detect antibody in pooled milk samples from dairy herds) and the market cattle identification blood test (to test serum antibodies in blood samples).

Serological tests used to detect *B. canis* in dogs include rapid slide agglutination (card or RSAT) tests, tube agglutination, an indirect fluorescent antibody (IFA) test, AGID and ELISA.

In swine, serology is generally considered to be more reliable for identifying infected herds than individual pigs. Serological tests used in swine include ELISAs, the buffered *Brucella* antigen tests and complement fixation. A fluorescence polarization assay has been developed. Supplemental serological tests used in cattle may also be used in swine (Garcia, 1990).

The serological tests used in marine mammals have been adapted from livestock *Brucella* tests. They include the buffered *Brucella* antigen tests, serum agglutination tests, complement fixation, AGID, ELISAs and rivanol test (Foster *et al.,* 2007). In general, these tests have not yet been validated for marine mammals; threshold values have not been established and can vary between laboratories.

## 2.6.4. Molecular techniques and other tests

Immunostaining techniques are sometimes used to detect *Brucella* antigens in tissue samples. A brucellin allergic skin test can be used to test pigs for *B. suis,* or unvaccinated small ruminants and cattle for *B. melitensis* or *B. abortus*, respectively. Polymerase chain reaction (PCR) techniques are also available for most species. rRNA sequencing has defined the phylogenetic relationship of *Brucella*. Its closest known relation, *Ochrobactrum anthropi*, is an environmental bacterium associated with opportunistic infections (Cieslak et al., 1992), this organism is also detected by a PCR that is otherwise specific for *Brucella* (Da Costa et al., 1996)*.* Possibly more closely related is the incompletely characterized *Vibrio cyclosites,* which displays >90% similarity of 5S rRNA sequence (Minnick and Stiegler, 1993). Less closely related but within the same subgroup of the -2 Proteobacteria are *Agrobacterium, Phyllobacterium,* and *Rhizobium,* which also possess multiple replicons and a capacity for intracellular growth. The *Bartonella* group also shows some affinity to *Brucella* on the basis of rRNA but not DNA similarity (Relman et al., 1992).

**2.7. Treatment**

Due to intracellular localization of Brucella and its ability to adapt to the environmental conditions encountered in its replicative niche e.g. macrophage (Seleem et al., 2008), treatment failure and relapse rates are high. There is no practical treatment for infected cattle or pigs, but long-term antibiotic treatment is sometimes successful in infected dogs. Some dogs relapse after treatment. Antibiotic treatment has also been used successfully in some valuable rams, but it is usually not economically feasible. Fertility may remain low even if the organism is eliminated. In horses with fistulous withers or poll evil, the infected bursa may need to be surgically removed. In regions with high prevalence of the disease, the only way of controlling and eradicating this zoonosis is by vaccination of all susceptible hosts and elimination of infected animals (Briones et al*.,* 2001).

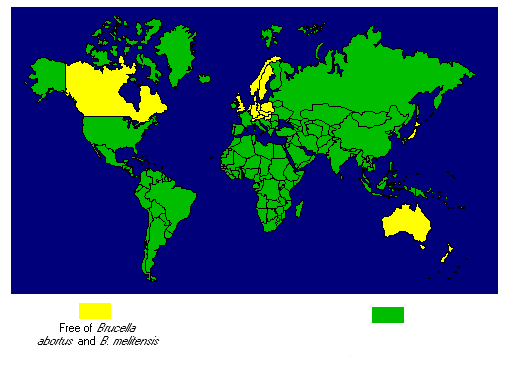
## 2.8. Prevention

Brucellosis is usually introduced into a herd or kennel in an infected animal, but it can also enter in semen. Herd additions should come from brucellosis-free areas or accredited herds. *B. ovis*-free accredited rams may be available in some areas. Animals from other sources should be isolated and tested before adding them to the herd. Domesticated animals should always be kept from contact with wild animal reservoirs. Commercial *B. abortus* and *B. melitensis* vaccines are available for cattle, sheep and goats. The most commonly used vaccines against bovine brucellosis are *B. abortus* strain 19 and the recently USDA approved strain RB51; the latter unlike strain 19 does not interfere with serological diagnoses (Moriyon *et al.,* 2004). Vaccination can interfere with serological tests; this is minimized when only young animals are vaccinated. Vaccination for *B. ovis* is practiced in New Zealand and some other countries, but not in the U.S. Successful vaccines have been difficult to develop for pigs; this species is generally not vaccinated except in China. No vaccines are made for dogs and human (Henk and Kadri, 2005). Nationwide eradication programs for *B. abortus*, *B. melitensis* and *B. suis* include quarantines of infected herds, vaccination, test-and-slaughter and/or depopulation techniques, cleaning and disinfection of infected farms, and various forms of surveillance and tracebacks. *B. ovis* has been eradicated from sheep in the Falkland Islands by test-and-removal methods directed at rams. In areas where a *Brucella* species is not endemic, infected herds are usually quarantined and the animals are euthanized. In the USA, *B. suis* has been eradicated from commercial swine, and *B. abortus* has nearly been eradicated from domesticated ruminants. Various control methods are being directed at wild animal reservoirs including wild bison and elk herds in the Greater Yellowstone Area, and wild and feral swine. Vaccination against brucellosis is prohibited in Romania and like many other developed countries they have eradicated *Brucella abortus* from cattle since 1969 (Dobrean et al., 2002). Brucellosis has been eradicated from many countries (Table 01 and Fig 01).

Table 1: Countries reporting eradication of bovine brucellosis’ 1994

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **EUROPE** | | **ASIA** | | **AFRICA** | | **AMERICAS** | | **OCEANIA** | |
| *Country* | *Year* | *Country* | *Year* | *Country* | *Year* | *Country* | *Year* | *Country* | *Year* |
| Bulgaria | 1958 | Cyprus | 1932 | Mauritius | 1986 | Belize | 1980 | Australia | 1989 |
| Croatia | 1965 | Israel | 1984 |  |  | Canada | 1989 | French Polynesia | 1984 |
| Czech Republic | 1964 | Japan | 1992 |  |  |  |  | New Zealand | 1989 |
| Denmark | 1962 | Jordan | 1992 |  |  |  |  | Vanuatu | 1992 |
| Estonia | 1961 | N Korea | 1959 |  |  |  |  |  |  |
| Finland | 1960 | Papua New Guinea | 1974 |  |  |  |  |  |  |
| Hungary | 1985 | Philippines | 1989 |  |  |  |  |  |  |
| Iceland | never recorded | U.A.E. | 1992 |  |  |  |  |  |  |
| Latvia | 1963 |  |  |  |  |  |  |  |  |
| Lithuania | 1952 |  |  |  |  |  |  |  |  |
| Luxembourg | 1993 |  |  |  |  |  |  |  |  |
| Netherlands | 1993 |  |  |  |  |  |  |  |  |
| Romania | 1969 |  |  |  |  |  |  |  |  |
| Slovak Republic | 1964 |  |  |  |  |  |  |  |  |
| Slovenia | 1970 |  |  |  |  |  |  |  |  |
| Sweden | 1957 |  |  |  |  |  |  |  |  |
| Switzerland | 1963 |  |  |  |  |  |  |  |  |
| U.K. | 1993 |  |  |  |  |  |  |  |  |

Source for Tables 1: FAO-WHO-OIE AnimalHealth . Yearbooks, 1994, 1995.



Brucellosis

suspected areas

Figure :Map showing countries that eradicated Brucellosis.

Specific control methods have not been established for brucellosis in marine mammals. General principles of infection control including isolation, disinfection and good hygiene should be practiced with infected animals. Some authors suggest that centers involved in marine mammal rehabilitation should routinely screen animals for *Brucella* (Foster et al., 2007).

## 2.9. Morbidity and Mortality

*B. abortus*, *B. melitensis* and *B. suis* are associated with a high morbidity rate in naive herds, and a much lower morbidity rate in chronically infected herds. In naive cattle, *B. abortus* spreads rapidly, and 30% to 80% of the herd may abort (Acha and Szyfres, 2003). In herds where this organism has become endemic, only sporadic symptoms occur and cows may abort their first pregnancies. A similar pattern is seen with *B. melitensis*-infected sheep and goats. Likewise, when *B. suis* is first introduced into a herd, there may be a significant increase in returns to service, abortions and stillbirths, weak piglets, lameness/ arthritis, posterior paralysis and other signs. The pre-weaning mortality rate usually increases. However, in endemic swine herds, brucellosis may appear as non-specific infertility, a slightly reduced farrowing rate, and irregular estrus cycles. In domesticated pigs, the abortion rate from *B. suis* varies widely, from 0% to 80% (Acha and Szyfres, 2003). Fertility can be permanently impaired after infection with some species of *Brucella*. Deaths are rare in adult animals of most species; however, *B. abortus* can be lethal in experimentally infected moose, and possibly in bighorn sheep.

*B. ovis* has little effect on sperm quality in some individual animals, but causes severe decreases in sperm motility, concentration and morphology in others. Approximately 30-50% of all infected rams have palpable lesions of the epididymis (Acha and Szyfres, 2003). Estimates of the abortion rate vary. Some sources report that *B. ovis* causes abortion and perinatal lamb mortality rates of 1–2%, while others suggest that these outcomes are rare. Limited experimental studies have reported abortion rates from 0% to 8%. Abortions and increased perinatal mortality have not been reported in red deer.

*B. canis* spreads rapidly in confined populations, particularly during breeding or when abortions occur. Although death is rare except in the fetus and neonate, significant reproductive losses can be seen, particularly in breeding kennels. Up to 75% fewer puppies may be weaned from affected kennels (Acha and Szyfres, 2003).

The morbidity and mortality rates for brucellosis in marine mammals are unknown (Foster et al., 2007).

## 2.10. Serological tests and prevalence study

Worldwide, brucellosis remains a major source of disease in humans and domesticated animals. Although reported incidence and prevalence of the disease vary widely from country to country, bovine brucellosis caused mainly by *B. abortus* is still the most widespread form (Tables 02).

**2.11.**  **Global Perspective.**

Brucellosis is an emerging disease since the discovery of *Brucella melitensis* as the cause of Malta Fever by Bruce in 1887 and the isolation of *B. abortus* from aborted cattle by Bang in 1897 (Nicoletti, 1990). The importance of brucellosis is not known precisely, but it can have a considerable impact on human and animal health, as well as socioeconomic impacts, especially in which rural income relies largely on livestock breeding and dairy products (Roth et al., 2003). Human brucellosis is caused by exposure to livestock and livestock products. Infection can result from direct contact with infected animals and can be transmitted to consumers through raw milk and milk products. In humans, the symptoms of disease are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly, and night sweats (Madkour, 2001). Recently, it has been reported that brucellosis can affect the central and peripheral nervous system of human (Al – Sous et al., 2004). There are so many factors that can affect the prevalence of brucellosis in various species of livestock. Prevalence of brucellosis can vary according to climatic conditions, geographical location, species, sexes, ages and diagnostic reagents, methods and tests applied (Gul and Khan, 2007).

Variable prevalence of brucellosis has been reported in sheep and goats. Bio varieties of *Brucella* vary with respect to geographical location. *Brucella melitensis* biovar 1 from Libya and Oman with prevalence of 4.1% and 8% respectively; biovar 2 from Turkey and Saudi Arab have been isolated with sero – prevalence of 2% and 8% respectively ( Memish, 2001). *Brucella melitensis* biovar 3 is the most commonly isolated species from animals in Egypt, Israel, Tunisia and Turkey (Refai, 2002). *Brucella abortus* biovar 1 in Egypt, biovar 2 in Iran, biovar 3 in Iran and Turkey, and biovar 6 in Sudan have been reported (Halling and Boyle, 2002). *Brucella abortus* has been isolated in cattle and buffalo from Egypt, Iran, Iraq and Sri Lanka with sero – prevalence of 10%, 0.85%, 3% and 4.7% respectively (Silva et al.,2000). The incidence of infection in reactor animals in newly established cattle farms may be more than 30%, however, the highest rate (72.9%) of infection till now has been reported in the Palestine Authority (Shuaibi, 1999). It is interesting to note that second highest sero – prevalence (71.42%) of brucellosis has been reported in the mules from Egypt (Anonymous, 2007). Sero – prevalence of brucellosis in cattle of Urmia region at Iran was very low as 1.17 – 1.22% (Hamid et al., 2011). Higher sero – prevalence reports are 39% in Western Ethopia (Mayer, 1980) while lower sero – prevalence was found by Omer *et al.* (2000) 8.2% in Eritrea and El – Ansary et al. (2001) 5% in Sudan.

Table : Brucellosis in animals, Asia, 1994.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Country** | **Animal and *Brucella* spp.** | | | |
| Bovine  *(B. abortus)* | Ovine/caprine  *(B. malitensis)* | Porcine  *(B. canis)* | Ovine  *(B. ovis)* |
| Afghanistan | + | + | ND | ND |
| **Bangladesh** | **+** | **+** | **ND** | **ND** |
| Bhutan | + | - | - | ND |
| China | + | + | + | + |
| Hong Kong | ND | ND | ? | ND |
| India | + | + | ? + | - |
| Indonesia | + | ND | + | + |
| Iran | + | + | - | - |
| Israel | - | + | - | - |
| Iraq | + | + | ND | ND |
| Jordan | - | ++ | - | - |
| Korea (S) | ++ | - | ? + | - |
| Kuwait | ++ | ++ | - | - |
| Malaysia | + | - | ? + | - |
| Mongolia | ++ | + | - | + |
| Myanmar | + | ND | + | ND |
| Oman | ++ | ND | ND | ND |
| Qatar | ND | ND | ND | ND |
| Sri Lanka | ++ | + | - | + |
| Syria | + | ND | ND | ND |
| Thailand | + | - | + | - |
| Turkey | ++ | ++ | - | ND |
| UAE | - | + | - | + |
| Yemen | + | + | - | - |

- not present

+ low sporadic incidence

++ high incidence

? presence uncertain

ND no data

None of the four types of brucellosis is present in Bahrain, Cyprus, Japan, Malaysia (Sabah), Philippines, or Singapore. No data for countries of the former Soviet Union or Qatar.

Source for Tables 2: FAO-WHO-OIE AnimalHealth . Yearbooks, 1994, 1995.

## 2.12. Local (Bangladesh) Perspective.

The economic importance and the sero – prevalence of brucellosis in man and animals have been reported form some parts of Bangladesh (Ahmed et al., 1992; Islam et al., 1983; Rahman et al., 1978; Rahman and Rahman, 1982; Rahman et al., 1983). In 1997, a study was carried out on sero – prevalence of brucellosis in buffalo of a selected area in Bangladesh, and in general, 6.9% buffaloes were positive to brucellosis (Rahman et al.1997). The sero – prevalence of brucellosis was determined in the ruminants like buffaloes, cattle, sheep and goats of five different districts viz. Bagerhat, Bogra, Gaibandha, Mymensingh and Sirajgonj of Bangladesh. A structured questionnaire was used to collect epidemiological information on the animals. The overall serological prevalence derived from the samples was 2.87% in buffalo, 2.66% in cattle, 3.15% in goats, and 2.31% in sheep respectively. The prevalence was relatively higher in female animals than in male animals (Rahman et al.,2011).

In 2005, a studied on cow for sero – prevalence of *Brucella* antibodies in Bangladesh and the results revealed that number of Red Shindi was the highest and the sero – prevalence of brucellosis in Bangladesh compared to indigenous population (Amin et al.,2005). A cross sectional survey was conducted of BAU Veterinary Clinics, BAU Dairy Farm and a village of Vabokhali from June to November 2008 (Rahman et al., 2009). A similar study of sero – prevalence of brucellosis was conducted on different species of animals with a history of abortion and found sero – positive 60% in buffaloes, 57.14% in cattle, 66.67% in goats and 50% in sheep respectively (Rahman et al.,2011).

## 2.13. Molecular Identification and Characterization of *Brucella*

Modern genetic characterizations of Brucellae using molecular DNA technology have been developed. Several PCR – based assays have been proposed, from the rapid recognition of genus to differential identification of species and strains (Poester et al*.,* 2010). Bricker et al.(2003) also evaluated the *Brucella abortus* species – specific polymerase chain reaction assay which is an improved version of the *Brucella* AMOS PCR assay for cattle, and they used 5′ - GTG-CCA-GCA-GCC-GCC-GTA-ATA-C-3′ and 5′ - TGG-TGT-GAC-GGG-CGG-TGT-GTA-CAA-G-3′ as 16S – universal forward primer and 16S – universal reverse primer respectively for *Brucella abortus*. Doosti and Dehkordi (2011) applied both conventional PCR and Real – time PCR for identifcation and differentiation of *Brucella abortus* and *B. melitensis* in cattle. Doosti and Dekhordi (2011) used 5'-GCG-CTC-AGG-CTG-CCG-ACG-CAA-3' and 5'-ACC-AGC-CAT-TGC-GGT-CGG-TA-3' as forward primer and reverse primer respectively for *Brucella abortus* and *B. melitensis*. Dehkordi *et al*. (2012) detected and segregated the *Brucella abortus* and *Brucella* *melitensis* in aborted bovine, ovine, caprine, buffaloes and camelid fetuses by application of Conventional and Real – time PCR. In conventional PCR, Dehkordi *et al*. (2012) used 5′ - GCG-CTC-AGG-CTG- CCG-ACG-CAA-3′ and 5′ - ACC-AGC-CAT-TGC-GGT-CGG-TA-3′ as forward primer and reverse primer respectively for *Brucella abortus* and *B. melitensis*.

## 2.14. Vaccine against bovine brucellosis

Fensterbank R. and Plommet N. (1979) studied over vaccination against bovine brucellosis with avirulent *Brucella abortus* Strain 19 was usually performed on 4 – 6 months old heifers, so that the post – vaccinal response which lasted 6 – 12 months did not disturb the serological tests on adults and they applied the vaccine on conjunctival route. Comerci *et al*. (1998) studied over a vector for the expression of foreign antigens in the vaccine strain *Brucella abortus* S 19 was developed by using a DNA fragment containing the regulatory sequences and the signal peptide of the *Brucella bcsp 3 1* gene. The first effective *Brucella* vaccine was based on *Brucella abortus* strain 19, a laboratory – derived strain attenuated by an unknown process during subculture which induced reasonable protection against *B. abortus* (Schurig *et al.*, 2002)*.*

The true prevalence of cattle brucellosis remains poorly estimated or unknown in many countries. Apparent prevalence of brucellosis is mentioned bellow:

**2.15. Prevalence**

**F. Khamesipour *et.al.,*2014**: The study was conducted to detect Brucella sp. and Leptospira sp. in blood samples of dogs in Isfahan and Shahrekord province in Iran. A total of 94 blood samples were collected from dogs of different breed, age, sex, and dogs’ type (stray or non-stray). The samples were examined using conventional polymerase chain reaction (PCR). Fourteen (14.89%) dogs were positive for Brucella sp. and 18 (19.15%). dogs for Leptospira sp. There were no significant differences between the prevalence of the pathogens, provinces, sex, and age groups (P > 0.05)

[**K. Karthik**](http://ascidatabase.com/author.php?author=K.&last=Karthik) ***at.al*., (2014):** A study aimed at to know the prevalence of B. abortus in cattle population of three states (Uttar Pradesh, Uttarakhand and Tamil Nadu) of India by serological (Rose Bengal Plate Test (RBPT) and Serum Tube Agglutination Test (STAT)) and molecular ([polymerase chain reaction](http://www.scialert.net/asci/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=polymerase+chain+reaction)) detection in sera samples and whole blood (n = 370), respectively. Out of a total of 370 sera samples, 61 (16.49%) were positive by RBPT and 59 (15.94%) by STAT. Screening of the whole blood samples by genus specific bcsp31 gene based PCR as well as species specific IS711 gene based PCR revealed that 56 (15.13%) samples were positive for brucellosis. None of the serologically negative sample showed positivity by PCR;

**M. Saberi at.al., ( 2013):** Tissue samples were taken from the abomasal fluid, liver, kidney, spleen, lung, heart and brain of the aborted fetuses and dam’s placenta and tested by PCR. Twelve out of 100 dams (12%) were seropositive to the Brucella spp. and twelve out of 100 aborted fetuses (12%) showed positive reaction to the Brucella melitensis Rev-1 vaccine strain by the PCR.

**Jabbar A.A 2012**: In this study, 59 aborted buffalos and 91 aborted cows were included. Diagnosis of Brucella infection in these abortions was based on clinical, serological, bacteriological, hormonal, and molecular assays. Serological studies included the use of RB and ELISA tests as screening tests for infection. Argumentative differences between RB and ELISA results have been shown. Brucella isolated and identified from aborted fetuses, vaginal discharge and milk samples were 7 isolates from aborted cows and 3 from aborted buffalos.

**Dehkordi1 F** ***et.al*.,** **(2012):** In Shahrekord, Iran study was carried out to use conventional and real-time PCR for detection and segregation of Brucella abortus and Brucella melitensis in aborted bovine, ovine, caprine, buffalo and camel fetuses. All samples were collected and immediately transferred to laboratory, genomic DNA was extracted and the conventional and real-time PCR by specific primers for Brucella abortus and Brucella melitensis was performed. A TaqMan analysis and single-step PCR was carried out in total 3710 DNA of abomasal contents of aborted fetuses. In total, 281/892 (31.5%) bovine, 224/810 (27.65%) ovine, 219/786 (27.86%) caprine, 199/604 (32.94%) buffalo and 201/618 (32.52%) camel fetus samples gave positive results for Brucella species by conventional PCR.

**Tuba ICA *et al.,(*2012)** Samsun, Turkey In this study, the role of Brucella spp. in cattle and sheep abortions among Kayseri region was investigated and predominant subspecies and biovars in this region were determined by conventional and molecular biotyping methods. For this purpose, 61 cattle and 64 sheep abortion material and also 50 human blood isolates were examined. A total of 29 Brucella spp. 17 (27.9%) and 12 (18.7%) of which were isolated from cattle and sheep specimens, respectively) were isolated from animal sources.Both animal and human isolates were typed by conventional.

**Doosti A. and Dekhordi P.G. (2011):** This study was performed to determine the prevalence of Brucella spp. and to differentiate Brucella melitensis and Brucella abortus in cattle population in southwest Iran. A TaqMan analysis and singlestep PCR was performed in total of 425 bovine blood samples. The results showed 127 (29.88%) positive samples for Brucella spp. By real-time PCR 9, 69, and 5 of these specimens, were positive for B. melitensis, B. abortus, and both bacteria respectively. Results of present study indicated a high presence of this pathogen in the region. Real-time PCR is technically more simple, accurate, and rapid than current standard methods for identification and differentiation of Brucella species

**Hamali H, &. Joozani R.J (2011**)**:** Tissue samples were taken from the stomach (fluid), liver, kidney, spleen, lungs, heart and placenta of aborted fetuses and tested by PCR. Six out of 76 dams (7.8 percent) were seropositive to the Brucella spp., and six out of 76 aborted fetuses (7.8 percent) showed a positive reaction to the PCR test. Four out of six aborted fetuses (66 percent) showed a positive reaction against the Brucella abortus and the two remaining (34 percent) had a positive reaction to the vaccine strain, RB51. Statistical analysis did not show any significant difference between the two diagnostic methods (PCR and serological tests).

**Bayemi *et al.* (2009):** Carried out a study on Holstein cattle of a small scale dairy production system, screened for Brucella abortus antibodies in 21 villages in Cameroon by ELISA. Of the 192 cows tested, 14 were infected within sex seroprevalence of 7.3% while 6/74 bulls were infected with a seroprevalence of 8%. There was no evidence (P = 0.11) of differences in seroprevalence between age groups although animals above one year and below three years accounted for nearly half of the infected animals. 64% of infected animals were found in three locations (P = 0.015): Kutaba (32%), Bamendankwe (16%) and Finge (16%).

[**Muma**](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Muma%20JB%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus) ***et al.* (2009):** Demonstrated the effectiveness of Rose Bengal test (RBT) and fluorescence polarization assay (FPA) in diagnosing cattle brucellosis in endemic areas and RBT and FPA test agreement was compared (n = 319). There were 79.3% agreement between the RBT and FPA (Kappa = 0.59; Std error = 0.05; p = 0.000) and a high correspondence between high RBT scores and positive FPA results suggesting that sera with high RBT score may not require confirmation with tests such as competitive-ELISA or CFT. High FPA cut-off points were more likely to miss animals with low antibody titres.

**Maher and Venkataraman (2008):** were conducted a study to compare the Rose Bengal test (RBT) and standard tube agglutination test (STAT) with i-ELISA in detecting Brucella melitensis antibodies in sheep in India. Out of 231 sera sample were tested for brucellosis by RBT, STAT and i-ELISA. 20.35, 13.86 and 9.96% sheep were found positive to B. melitensis by i-ELISA, RBT and STAT, respectively. The sensitivity and specificity of RBT with i-ELISA were 53.19 and 96.19%, respectively. The sensitivity and specificity of STAT with i-ELISA were 42.55 and 98.37% respectively.

**Simone Miyashiro at.all., (2007):** A total of 192 samples of illegal cheese from different regions of the states of São Paulo and Minas Gerais, Brazil, were analyzed for the isolation and detection of *Brucella* spp. DNA by means of microbiological culture and polymerase chain reaction (PCR), respectively. Samples that yielded positive results were submitted to the analysis of the occurrence of *Brucella abortus* (biovars 1, 2 e 4), as well as to the differentiation of DNA in B19 vaccinal strain or *Brucella abortus* field strain using PCR. Although the microorganism was not isolated from any sample, PCR detected 37 positive samples (19.27%) using genus-specific primers.

**Apan** et al. **(2007):** Conducted a study on the seroprevalence of brucellosis in human, sheep and cattle populations in rural regions of Krkkale in Turkey. Serum samples were obtained from 1436 humans, and 3.2 % (46) of the population found to be positive by Rose Bengal Plate test (RBPT) and 3.0 % (43) gave positive results with the Standard Tube Agglutination Test (STAT).

**Berhe et al. (2007):** While studied at Northern Ethiopia, observed that age and herd size did not significantly affect brucellosis seropositivity. Animals more than 3 years of age had a higher brucellosis seroprevalence compared to those aged 0.6-3 years. A higher seroprevalence was recorded in farms having 21-50 animals (0.30%), followed by those with 1-20 animals (0.30%). Farms with more than 50 animals were negative for antibodies. The highest prevalence was recorded in single parity animals (0.53%), followed by those with multiple parities (0.39%) and no parity (0%).

**Hussein et al. (2007):** Reported that out of 175 unvaccinated cattle 84 (48%) were shown positive agglutination reaction towards Brucella melitensis while 44 (26.4%) showed positive reaction toward Brucella abortus. The highest incidence of infected animals and mix infection were recorded in cows with more than 10 years old. Cows milking between 6-10 times showed highest incidence of the infection in Aaaiur Governoate, Egypt.

**Muma** et al. **(2007):** Observed in his study that out of three serological tests for brucellosis; Rose Bengal Test (RBT), Competitive ELISA (c-ELISA) and Fluorescence Polarisation Assay (FPA) and none of the tests can be seen as a perfect reference test or gold standard. The highest sensitivity (Se) was achieved by the c-ELISA (97%; Credible Posterior Interval (CPI) =93-100%) and the highest specificity (Sp) by the FPA (93%; CPI=85-99%), conversely these tests also had the lowest Sp and Se, respectively, with the RBT performing well in both the Se (93%; CPI=84-98%) and Sp (81%; CPI=61-97).

**Radulescu** et al. **(2007**): Observed in his study that the complement fixation test had the highest specificity and sensibility, followed by Rose Bengal Test and SAT. The most efficient serodiagnostic tool is a combination of the three assays. The use of only one assay for the diagnosis of brucellosis is not recommended.

**Robles et al. (2007):** Carried out a study to determine the prevalence of brucellosis in goats from the Mendoza Province, Argentina. 8377 serum samples belonging to 566 farms were obtained. BPA was used as the serological test. 28.1% of the farms were positive with prevalence ranging from 6.7% to 80%. The 5.7% of the processed sera were positive to BPA.

**Vikrant et al., (2006)** screened serum samples from 186 cattle, 45 buffaloes, 56 sheep and 168 goats for brucellosis by STAT, RBPT and ELISA between January and May 2003 in India. The seroprevalence was recorded in sheep (16.07%), followed by cattle (7.53%), goats (7.14%) and buffaloes (6.67%). The disease was more prevalent in female (9.38%) than male (5.92%) animals.

**Rahman et al. (2006)** reported the seroprevalence of brucellosis as 2.4%-18.4% in cattle while the herd-level seroprevalence of brucellosis as 62.5% in cattle in Bangladesh.

**Subash et al. (2006):** conducted a survey of brucellosis in cattle and buffaloes of organized sector (Goshala and Tabela) and unorganized sector at Jodhpur region, India. The Rose Bengal plate Test (RBPT) was used for rapid screening of sera and samples giving a positive reaction were subjected to the serum tube agglutination test (STAT). A total of 859 cattle and 133 buffaloes were sampled. The results of serum tube agglutination test on sera which had given to a positive reaction to the screening test indicate the presence of brucellosis is approximately 41.79% in cattle and 25.56% in buffaloes. The prevalence of Brucellosis was more in cattle of organized sector (Goshala) in comparison to unorganized sector.

**Kuralkar et al. (2006**): observed the prevalence of brucellosis in an organized dairy farm in Maharashtra, India where a total of 1167 (176 males and 991 females) crossbred cattle above 6 months of age were tested for brucellosis from 1989-2000. 29 animals (2.48%) were positive for antibodies against *Brucella* spp. Over the period of study, 672 calving were obtained and 13 abortions also occurred. 32 cows had placental retention while 4 cows produced stillborn calves.

**Sharma et al. (2006)** reported on his study that out of the 531 serum samples from sheep and goats tested by dot-ELISA, 46 were positive. The overall prevalence of brucellosis was 8.66% in India. With RBPT, 35 samples yielded agglutinins, resulting to a prevalence of 6.59%. Of the 439 goat serum samples, 28 and 21 (6.37 and 4.78%) were positive for brucellosis by dot-ELISA and RBPT, respectively. Among the 92 sheep serum samples, 18 and 14 were positive for brucellosis by dot-ELISA and RBPT, respectively (19.56 and 15.21%, respectively). In goats, the sensitivity and specificity of RBPT compared to dot-ELISA were 67.85 and 99.51% and in sheep, the values were 55.55, 94.59 and 86.95%, respectively.

**Amin et al. (2005)** demonstrated that the higher rate of *Brucella* antibody was recorded in rural farms (5.0%) than in organized farms (2.5%) and in pregnant cows (5.9%) than non-pregnant cows (4.7%). A total of 3 (4%) *Brucella* positive antibody cases were recorded in >4-year-old cows and 1 (2.3%) positive case was found in <4-year-old cows. The study was conducted in sera of 120 cows in Bangladesh Agricultural University Dairy Farm and adjacent villages and revealed that prevalence of brucellosis was highest in Red Shindi.

**Asghar et al. (2005):** demonstrated the seroprevalence of ovine brucellosis during rituals in India. Out of 374 sera samples of sheep, 29 samples were positive in the RBT, with a seroprevalence of 7.75%. 43 samples were positive in ELISA. 24 of these samples were also positive in RBT. The ELISA had comparable sensitivity and specificity to RBT but detected more positive samples than RBT. The low positive predictive value of ELISA in this study was probably a reflection of the lower specificity of the test compared to RBT.

**Bhattacharya et al. (2005)** conducted a study to determine the prevalence of brucellosis in the Livestock Research Station in Mandira and in the unorganized dairy farm of Assam, India, by using Avidin Biotin ELISA (AB-ELISA), Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination test (STAT). The seroprevalence was found to be significantly (P<0.05) lower in well-managed farms than unorganized farms. Seroprevalence was higher in cattle (14.28%) in comparison to buffaloes (9.73%). A higher incidence of brucellosis was recorded in aborted cows (55.00%) followed by repeat breeders (24.0%) and cases with retention of placenta (20.0%). Overall seroprevalence of brucellosis was found to be 11.94% and increased with age.

**Genc et al., (2005)** conducted a study to determine the seroprevalence of brucellosis and leptospirosis in aborted dairy cattle in Turkey. Serum samples were collected from 163 aborted dairy cattle that have no history of vaccination against either brucellosis or leptospirosis. The antibodies against *Brucella abortus* were detected in these serum samples as 68.1, 65.6, 58.9 and 55.2%, respectively, by the competitive enzyme linked immunosorbent assay (C-ELISA), complement fixation test (CFT), Rose Bengal plate test (RBPT) and serum agglutination test (SAT).

**Swai et al. (2005)** estimated the prevalence of antibodies against *Brucella abortus* infection in cattle by serum agglutination test (SAT) in April 2003 in Moshi, Tanzania. Sera were obtained from 417 dairy/local cattle of all ages, sexes and breeds that were kept in 113 randomly selected smallholder farms. The majority of the cattle were kept under zero grazing regimes. The overall prevalence of antibodies to *B. abortus* was 12.2 and 41.9% for individual cattle and farms, respectively. The true base on the age seroprevalence profile was estimated at 32.2 per 100 cattle years-risk.

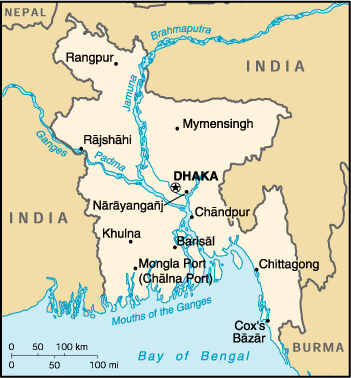
**Kuroda et al. (2004)** evaluated that out of 1789 cattle sera belonging to 96 herds submitted to Rose Bengal plate test (RBPT), agglutination test (AT), 2-mercapthoethanol test (2-ME) and complement fixation test (CFT). The regional prevalence at Botucatu mountains microregion in Brazil was 3.6% and 42.7% of the herds had at least one positive animal.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Site selection:

This study was conducted by the Department of Medicine and Surgery, Chittagong Veterinary and Animal Sciences University. This research was the continuation of an ongoing project of Prevalence study of bovine abortion. At least 25 sero-positive dairy farms were selected from Chittagong district in Bangladesh during the study period of one year (July 2013 to july 2014). Probable risk factors were investigated through molecular study of brucellosis which was previously identified as sero-positive. The following points were considered for this study.



Study area

Figure : Study area map

3.2. Farm selection: Twenty­-five large intensive private dairy farms were selected from the metropolitan and peri-urban area of Chittagong district (Annex-ii) which previously identified as sero-positive. The farm level includes the socio-economic conditions of the farm, stock inventory of the farms and steps were taken for managing the farm. Screening of farm and cows were based on previous history of abortion, stillbirth, agalactia, birth of weak calves and retention of fetal membrane (H.A. Kaoud et al. 2010). Samples like aborted fetuses were collected from the productive dairy cows of these selected dairy farms.

3.3 Aborted fetal sample collection and preservation:A total of 25 numbers of aborted bovine fetuses were collected, preserved at-300C until tests. The inoculums from eyeball, liver, kidney and lungs of each fetus was harvested for culture of Brucella after post-mortem of fetus at PRTC laboratory, CVASU and stored at -300C until use.

## 3.4 Procurement of reagent for culture of *brucella* organism:

A selective agar base and broth were procured before preparation of inoculums from eyeball, kidney, lung and liver.

a. Collumbia agar base (BBL, UK)

*b.Brucella* selective supplement (oxiod, UK) and

c.Horse serum (Hi-medium, India)

d. 5% co2 incubator used for preparation of solid media for culture of *Brucella abortus*.

## 3.5 Procurement of reagent for PCR of *brucella* organism:

a**.** FABGK001 (50preps) DNA extraction kit

b**.** *Brucella abortus* specific primer

c. Ladder of 50bp.

d. An ethidium bromide-stained agarose gel

All reagents supplied by the manufacturer company were preserved according their guideline.

## 3.6 Post- mortem for Harvesting of specimen from aborted fetuses:

A four number of specimen from eyeball, liver, kidney and lungs of each fetus was harvested for culture of *Brucella* after post-mortem of fetus at PRTC laboratory, CVASU and stored at -300C until use. Pieces from internal tissues of aborted fetuses were collected with set of sterile forceps and scissors and flamed after plunged to ethanol (Esra and Sen, 2007). Each specimen were used as inoculum for culture in artificial growth medium. Columbia Agar Base was used for growth of *Brucella.*

## 3.7 Inoculums preparation from specimen:

A total of 100 ( 25 x 4=100) innoculums were prepared from 25 harvested sample. Morter and pastle were autoclaved for sterilization and Bunsen burner for loof sterilization were done. Then the specimen were cutted into small pieses and massarate to prepared the innoculums. The innoculums were stored at -300C until use.

Fig: aborted fetus collected from sero-positive dam after abortion.

## 3.8 Preparation of culture medium for *Brucella abortus* :

The culture media was prepared from Columbia Agar Base (BBL), *Brucella* Selective Supplement (OXOID) and Horse Serum (OXOID), and described as follows:

Twenty two grams of Columbia Agar Base (BBL) was suspended in 500 ml of distilled water in a sterile beaker. This was heated to boil and dissolved the medium completely. Then it was sterilized by autoclaving at 15 lbs pressure, 1210C for 15 minutes. It was added after cooling the media to Brucella Selective Supplement (Chapin and Doven, 1983). A total of 10 ml Brucella Selective Supplement (OXOID) was mixed with 1:1 ratio of methanol and distilled water and mixed properly. Then it was incubated at 370C for 15 minutes. This sterile medium was mixed well and added to Columbia Agar Base. A total of 10% of 10 ml inactivated horse serum (OXOID) and 5% of 10 ml sterile dextrose solution was added to the medium and mixed well. Then 15 ml of medium was dispensed in to each sterile Petri dish. All sterile Petri dishes were incubated at 370C (United States Pharmacopeia, 2007). Few colonies was then transferred into 50% glycerin and kept at – 200C.

## 3.9 Inoculation of *Brucella abortus* on culture media:

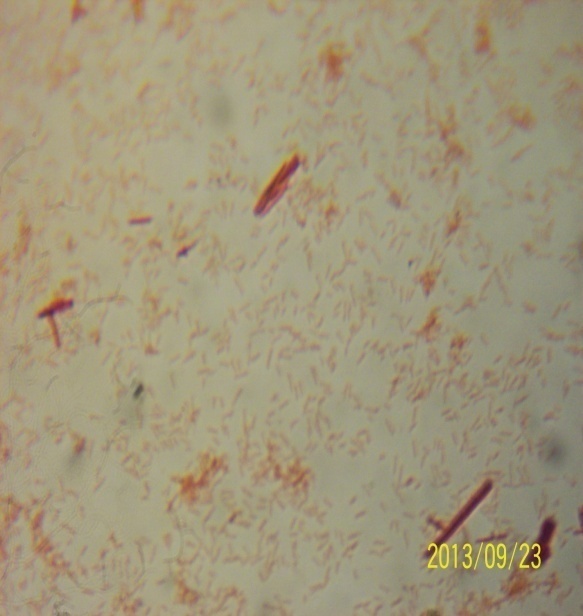
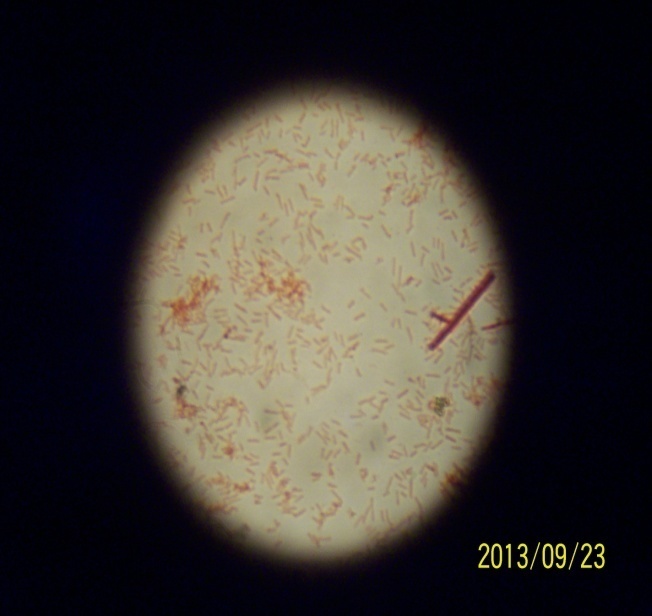
The aseptically collected specimens were inoculated on culture media of *Brucella* for cultivating and isolating the *Brucella abortus*. A 5% CO2 incubator was used for growth of *Brucella* at 370C for 24-48 hours.The result was recorded and incubated the positive at room temperature (2o-240C) in dark environment for 4-6 weeks.

****

Figure : Growth on culture media

## 3.10 Microscopic examination

Microscopic examination of smears stained with the Stamp's modification of the Ziehl-Neelsen method was used for a presumptive diagnosis (Joshi et al., 2005). Organisms was found in aborted products. *Brucell*a species are not truely acid-fast, but they are resistant to decolorization by weak acids, and stain red against a blue background. Brucellae are coccobacilli or short rods, usually arranged singly but sometimes in pairs or small groups.

****Figure : Microscopic examination

## 3.11 DNA extraction for conducting PCR in Kit Method :

DNA of *brucella spp* was extracted by using FABGK001 (50preps) DNA extraction kit by using the following protocol: Initially 200µl sample (prepared from inoculums) was transferred to a micro centrifuge tube. Then 20µl Proteinase K and 200µl FABG Buffer were added separately to the sample and mixed thoroughly by pulse vortexing and allowed to incubate at 60oC for 15 minutes to lyse the sample. During incubation sample was vortexed every 3-5 minutes. After that, 200µl absolute ethanol was added to the sample and thoroughly mixed it by pulse-vortexing for 30 seconds.

Followed by, FABG Column was placed to a collection tube and sample mixture (including any precipitate) was carefully transferred to FABG Column. Centrifugation was done at 1500 rpm for 1 minute and the flow-through was discarded then FABG Column was placed to a new Collection tube. Immediately, FABG Column was washed with 500µl W1 Buffer (ethanol added) then centrifuge 1500 rpm for 1 minute and discards the flow-through. The FABG Column was then washed with 750µl Wash Buffer by centrifuge for 1 minute then again the flow-through was discarding. Centrifugation was done for an additional 3 min to dry the column.

The FABG Column was then placed to the Elution Tube and 200 µl of Elution Buffer was added to the membrane center of FABG Column. Followed by FABG column was allowed to stand for 3 min for effective elution. Finally, Centrifugation was done for 2 min to elude the DNA and DNA fragment was Stored at -20oC until use.

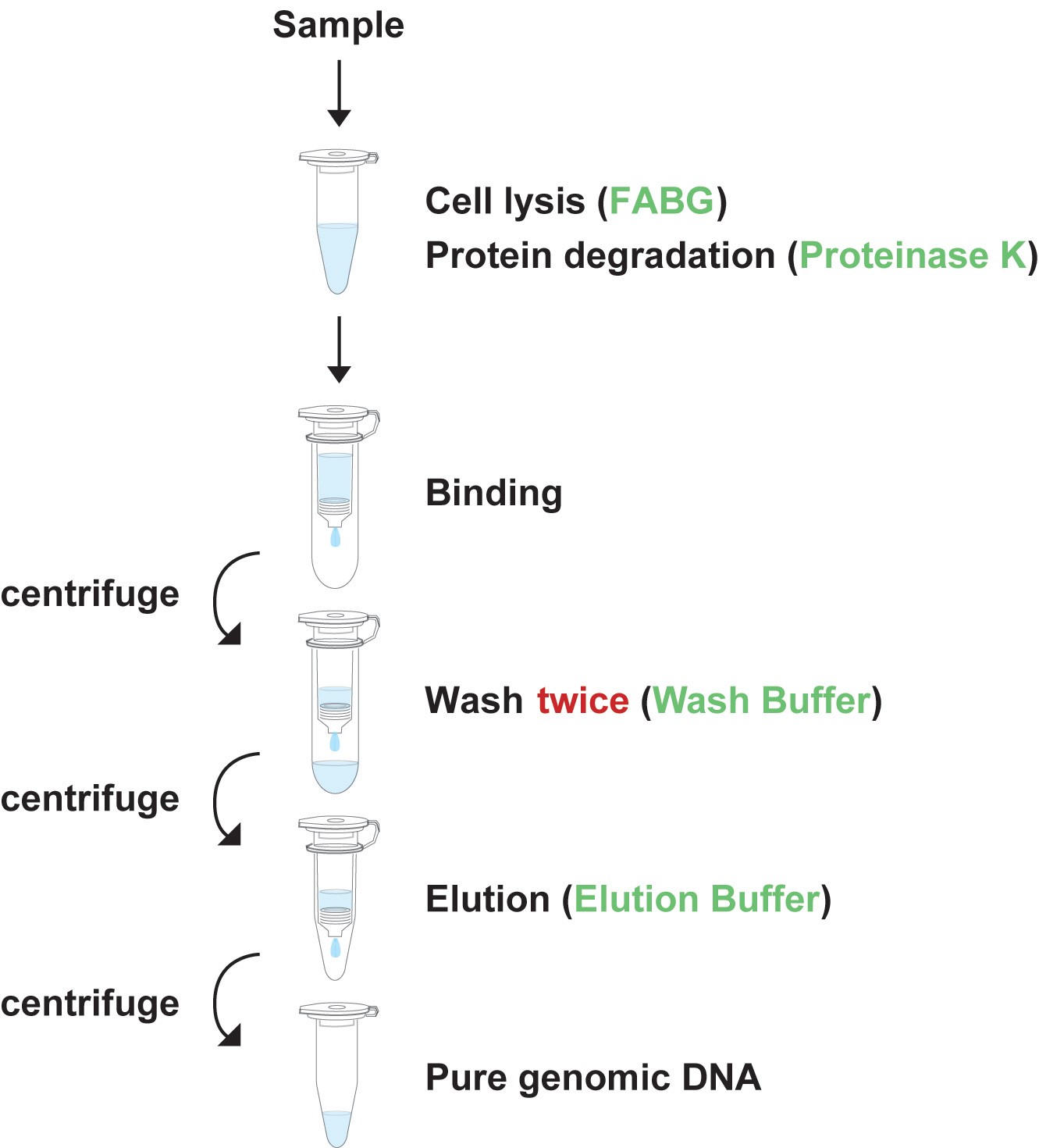


Figure : Brief procedure of DNA extraction by using DNA extraction kit.

## 3.12 PCR protocol

PCR was performed in a touchdown thermocycler in a total reaction volume of 50 ml containing 5 ml of 10xPCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM MgCl2, 1% Triton X-100), 250 mM each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega), 10 pg each of the primers derived from the rrs (16S) gene of *Brucella abortus*, forward primer A, 5’-GCG-CTC-AGG-CTG-CCG-ACG-CAA-3’ and reverse primer B, 5’-ACC-AGC-CAT-TGC-GGTCGG-TA-3 and 5 ml of template sample DNA. Parameters used were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing 50oC for 45 seconds, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Then the PCR product was store in 4° C temperature upto gel-run.The products (5 microlitres from each reaction mixture) will be analysed by electrophoresis through a 1.5% agarose gel for 1 hour at 100 volts (V) with 0.5×TBE buffer (89 mM Tris-HCl, 89 mM bori acid, 2 mM EDTA, pH 8). Gels was stained with ethidium bromide.

## 3.13 Gel preparation and gel-run:

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel for 1 hour at 100 volts (V) with 0.5×TBE buffer (89 mM Tris-HCl, 89 mM bori acid, 2 mM EDTA, pH 8). The gel was stained with 0.1% ethidium bromide (0.4 μg/mL) and viewed on UVIdoc gel documentation systems (UK).

## 3.14 Band identification and taking photogrape:

Bands of DNA fragment was detected with a UV transilluminator, and photographed with a digital camera. An ethidium bromide-stained agarose gel of PCR products that shows the sensitivity of the assay. DNA marker (50bp); band at 117bp.

## 3.15 Analysis:

All tested results were documented in Microsoft XL and calculator for determining the percentage of sero – positivity, farm level risk factors, and cow level risk factors. Risk factors for farm level were farm owner qualification, total number of employee in the farm, types of feed or ration offered at the farm, source of semen etc. Risk factors for cow level were lactation number, breed group, BCS group, lactation status, etc. were analysed

# CHAPTER 4

# RESULT

In this study, 25 fetal samples of cattle from 25 sero-positive dairy farm of Chittagong district were tested for *Brucella* spp. A total of 5 fetal samples from 25 aborted fetuses (20%) were found positive for *Brucella spp.* in both technique growth on culture medium and conventional PCR method by using specific primer.

## 4.1 Cultural finding:

The aseptically collected 25 fetal samples (25x4=100 specimens) of aborted fetuses were inoculated on culture media for cultivating and isolating the *Brucella spp*. Though the samples were collected from previously identified sero-positive dairy farm but the culture growth indicated only 20% specimens were positive for *Brucella spp*. **(Table 03)**. On Gram’s stain in *Brucella spp.* was found as Gram negative coccobacilli with short chain or small groups.

Table : Growth of Brucella spp. from aborted fetuses on Columbia agar/Peptone broth base

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Specimen No. | Growth on Columbia agar base with Brucella selective supplement | | | | Results on Gram Stain and Remarks |
| Eyeball | Liver | Lung | Kidney |
| F1  (Fetus 1) | Growth after 24 hours | Growth after 72 hours | Growth after 72 hours | Growth after 72 hours | Positive for *Brucella spp.* |
| F11  (Fetus 11) | Growth after 24 hours | Growth after 96 hours | No Growth | No Growth | Positive for *Brucella spp.* |
| F14  (Fetus 14) | Growth after 48 hours | Growth after 96 hours | No Growth | No Growth | Positive for *Brucella spp.* |
| F24  (Fetus 24) | Growth after 48 hours | No Growth | Growth after 96 hours | Growth after 72 hours | Positive for *Brucella spp.* |
| F25  (Fetus 25) | Growth after 24 hours | Growth after 72 hours | Growth after 72 hours | No Growth | Positive for *Brucella spp.* |

## 4.2 PCR finding:

All the specimen which were positive on the cultural growth were also positive on conventional PCR technique. An ethidium bromide-stained agarose gel of PCR products were showing the sensitivity of the assay where DNA marker 100bp and band at 117bp **(Table 04** and **Figure)**.

Table :PCR result for Brucella spp. from aborted fetuses

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fetus ID** | **Eyeball** | **Liver** | **Lung** | **Kidney** | **Pooled sample** | **Remarks** |
| F1 (Fetus 1) | +ve | +ve | -ve | -ve | +ve | +ve (117bp) |
| F11 (Fetus 11) | +ve | +ve | -ve | +ve | -ve | +ve (117bp) |
| F14 (Fetus 14) | +ve | -ve | +ve | -ve | +ve | +ve (117bp) |
| F24 (Fetus 24) | -ve | -ve | +ve | +ve | -ve | +ve (117bp) |
| F25 (Fetus 25) | +ve | +ve | -ve | -ve | +ve | +ve (117bp) |

Figure : An ethidium bromide-stained agarose gel of PCR products were showing the sensitivity of the assay where DNA marker 50bp and band at 117bp.

## 4.3 Prevalence according to cultural growth:

Sero-positive fetal samples were cultured on agar medium through incubation and found different result according to organ.On Columbia agar medium, eyeball (20%), liver (16%), lung (12%) and kidney (8%) were positive for *Brucella spp.* **(Table 5)**.

Table : Prevalence according to cultural growth

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Number of incubate sample** | **Culture positive** | **Percentage** |
| Eyeball | 25 | 5 | 20% |
| Liver | 25 | 4 | 16% |
| Lung | 25 | 3 | 12% |
| Kidney | 25 | 2 | 8% |

## 4.4 Prevalence according to PCR:

PCR is a confirmation technique for the entire infectious agent. The organism *Brucella spp.* varies from organ to organ that is illustrated in (**Table 06)**.

Table : Prevalence according to PCR

|  |  |  |  |
| --- | --- | --- | --- |
| **Organ** | **Number of positive sample** | **PCR positive** | **Percentage** |
| Eyeball | 05 | 04 | 80% |
| Liver | 05 | 03 | 60% |
| Lung | 05 | 02 | 40% |
| Kidney | 05 | 02 | 40% |
| Pooled sample | 05 | 03 | 60% |

Figure : Comparative sensitivity and specificity of positive sample

## 4.5 Comparative sensitivity and specificity of all samples:

A total of 25 aborted fetuses were collected from the sero-positive dairy farms. After collection, inoculums were made from eyeball, liver, lung, kidney and pooled sample. Then the inoculums were cultivated, incubated and isolated where 13% were showing positive on culture medium and 11.2% positive on PCR technique for *Brucella* *spp*. **(Table 5)**.

Table : Comparative sensitivity and specificity of all samples

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Technique** | **Total no. of samples** | **Total no. of innoculums** | **Positive for *Brucella spp*** | **Percentage (%)** |
| Growth of culture medium | 25 | 100 | 13 | 13 |
| PCR Technique | 100 | 11 | 11 |

**4.6. Prevalence of abortion in different trimester**

## Table 8: Period of abortion among the seropositive cows during the gestation period

|  |  |  |
| --- | --- | --- |
| Stage | No of cases | Prevalence (%) |
| 1st trimester | 3 | 12 |
| 2nd trimester | 16 | 64 |
| 3rd trimester | 6 | 24 |
| Total | 25 |

The number of cases during the 1st, 2nd and 3rd trimester was 3, 16 and 6 respectively with the prevalence of 12%, 64% and 24% corresponding to the gestation. Prevalence was varied significantly among the stages. The highest prevalence found at second trimester of pregnancy, followed by third trimester and lowest prevalence was found during the first trimester of pregnancy.

# CHAPTER 5

# DISCUSSION

Brucellosis is a known threat for both human and animal health in many countries. According to Refai (2002), prevalence of brucellosis varies from region to region throughout the country. However this infection is mostly found in developing Mediterranean and Middle Eastern countries.

PCR technique is now well recognized and easy to do, highly sensitive and more specificity for detection of microorganisms. PCR are promising alternatives for the difficult culturing and identification of *Brucella spp.* via conventional methods. The results showed that PCR is a sensitive and specific method for detection and differentiation. PCR assays have been developed for the detection of *Brucella spp.* in a wide diversity of clinical samples such as aborted fetuses was observed by Buyukcangaz, 2011.

The aseptically collected specimens will be inoculated on culture media of *Brucella* for cultivating and isolating the *Brucella abortus*. Microscopic examination of smears stained with the Stamp's modification of the Ziehl-Neelsen method can be used for a presumptive diagnosis of Brucella abortus (Joshi et al., 2005). Organisms were found in aborted products, vaginal discharges, milk, semen or various tissues. *Brucella* species are not truely acid-fast, but they are resistant to decolorization by weak acids, and stain red against a blue background. Brucellae are coccobacilli or short rods were identify (Al Dahouk *et al.,* 2003) which is found similar to this study.

In this study, the prevalence of brucellosis in abortus cases of cattle was investigated. It was determined by cultural and molecular techniques (PCR) and the prevalence of brucellosis in abortus cases of cattle was detected as 20%. This result was nearly similar with the study of Simone Miyashiro et al., (2007), Cortez *et al*., (2006) in two different studies at Sao Paulo and Minas Gerais, Brazil Simone Miyashiro et al., (2007), were analyzed for the isolation and detection of *Brucella* *spp*. by means of microbiological culture and polymerase chain reaction (PCR), respectively. PCR detected 37 positive samples (19.27%) using genus-specific primers. And another is almost similar to a recent PCR-based study that included four different agents and resulted in an etiologic diagnosis in 22.6% of the cases (Cortez et al., 2006).

Table :Lower rate of prevalence reported by several scientists in different time and country compared to present study (20%)

|  |  |  |
| --- | --- | --- |
| **Name of Authors and study year** | **Prevalence (%)** | **Country or region** |
| [K. Karthik](http://ascidatabase.com/author.php?author=K.&last=Karthik) **et al. (** 2014) | 15.90 | Uttar Pradesh, India |
| [K. Karthik](http://ascidatabase.com/author.php?author=K.&last=Karthik) **et al.** (2014) | 14 | Uttarakhand, India |
| [K. Karthik](http://ascidatabase.com/author.php?author=K.&last=Karthik) **et al.** (2014) | 14 | Tamil Nadu, India |
| A.A.Jabbar et al. (2012) | 13.18 | Turkey |
| Ibrahim et al. (2009) | 15.00 | Jimma zone, Ethiopia |
| Muma *et al.* (2006) | 14.10 | Blue Lagoon, Zambia |
| Hunduma and Regassa (2009) | 11.20 | Shewa zone of Oromia region, Ethiopia |
| Abbas and Aldeewan (2009) | 10.00 | Iraq |
| Eshetu et al. (2005) | 10.00 | Addis Ababa, Ethiopia |
| Bhattacharya et al. (2005) | 14.28 | Assam, India |
| Vikrant et al. (2005) | 7.53 | Garhwal of Uttaranchal, India |
| Dhand et al. (2005) | 12.09 | Punjab, India |
| Schelling et al. (2004) | 7.00 | Canada |
| Singh et al. (2004) | 14.70 | Punjab, India |
| Sarumathi et al. (2003) | 12.50 | Andhra Pradesh, India |
| Miranda et al. (2003) | 11.11 | State of Parabia, Brazil |
| Refai (2002) | 10.18 | Iran, near east region |
| Iyisan et al. (2000) | 11.40 | Turkey |

In this study a total of 25 fetal samples were collected and tested by means of microbiological culture and polymerase chain reaction (PCR) which consistent with the method and technique and found 20% positive which is lower than Doosti A. and Dekhordi P.G. (2011) who established 29.88% in Shahrekord, Iran. In this study the prevalence of *Brucella spp.* (20%) was higher than that reported by Jabbar et al. (2012) in Turkey and  [Karthik](http://ascidatabase.com/author.php?author=K.&last=Karthik) **et al*.* (**2014) in Uttar Pradesh, India, who reported 13.18% and 15.90% in a sero-epidemiological survey respectively.

Table : Higher rate of prevalence reported by several scientists in different countries compared to present study. (20%)

|  |  |  |
| --- | --- | --- |
| **Name of Authors and study year** | **Prevalence (%)** | **Country or region** |
| S. [Ali](http://www.ncbi.nlm.nih.gov/pubmed?term=Ali%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23868548) et al. (2014) | 56.7 | Potohar Plateau, Pakistan |
| Dehkordi1 *F.S* et al. (2012) | 31.5 | Shahrekord Iran |
| ICA Tub et al. (2012) | 27.9 | aSamsun, Turkey |
| Ahmed et al*.* (2010) | 42.00 | Libya |
| Gebretsadik et al*.* (2007) | 42.30 | Tigray region, Ethiopia |
| Muma et al. (2006) | 28.00 | Lochinvar, Zambia |
| Subash et al*.* (2006) | 41.79 | Jodhpur, India |

It is readily perceived geographical variation in the sero-prevalence may reflect differences in the levels of natural immunity, management and husbandry practices employed, and sensitivities and specificities of the diagnostic methods used among researchers as well as genetic variation in disease resistance among the breeds maintained in the systems (Omer ***et al*.,** 2000 and Martinez et al., 2008).

Large Intensive Private Dairy Farms from seven divisions of Bangladesh were selected. In recent study 15 Farms from Chittagong division were selected on the basis of baseline survey and estimate the loss of calf crop. The farm level includes the socio – economic conditions of the farm, stock inventory of the farms, and steps to be taken for managing the farm. Screening of farm and cow were based on previous history of abortion, stillbirth, agalactia, birth of weak calves and retention of fetal membrane (Moller et al.,1967). Samples like blood, urine and aborted fetus were collected from the productive dairy cows of these selected dairy farms.

[Karthik](http://ascidatabase.com/author.php?author=K.&last=Karthik) **et al.** (2014) conducted a study aimed at to know the prevalence of *B. abortus* in cattle population of three states (Uttar Pradesh, Uttarakhand and Tamil Nadu) of India by serological (Rose Bengal Plate Test (RBPT) and Serum Tube Agglutination Test (STAT) and molecular (PCR) detection in sera samples and whole blood (n = 370), respectively. Out of a total of 370 sera samples, 61 (16.49%) were positive by RBPT and 59 (15.94%) by STAT. Screening of the whole blood samples by genus specific *bcsp31* gene based PCR as well as species specific *is711* gene based PCR revealed that 56 (15.13%) samples were positive for brucellosis. None of the serologically negative sample showed positivity by PCR; however few positive samples were tested negative by PCR.

Prevalence of brucella in different area of Bangladesh is confirmed through epidemiological study, clinical signs and serological study. The prevalence rates found by several scientists are not same according to region. Another reason is that the parameter sample, sample size, collection procedure, methodology are different from study to study. Sikder S et al. (2011) found in Chittagong 5% positive. Rahman and Rahman (1982) reported 11.52%, 2.92% and 2% in Pabna, Faridpur and Bogra districts of Bangladesh respectively and Rahman et al. (1978) found 11.44% and 4.19% in Savar and BAU dairy farm correspondingly. The higher prevalence in this study is might be due to geographical and managemental discriminations, farmers’ illiteracy about the disease and under nutritional stress combined by the lower immunity that developed following acute infection (Radostits et al.,2000)

# CHAPTER VI

# CONCLUSION

The results of current study propose that PCR is highly sensitive and specific for identification and differentiation of *Brucella* spp*.* and that it could be a suitable tool for diagnosis of brucellosis. The results presented high presence of *Brucella* spp. in cattle samples and the conclusions of present study suggested that control and eradication programs very much for prevent and reduce the economic the loses of brucellosis it seems to be necessary. Consequently to, it is essential to screen in all area frequently to prevent the spread of the disease and laboratory support is an significant tool in the diagnosis of the disease. Seemingly, PCR is one of the greatest ways to detect and characterize *Brucella* spp. as fast, less hazardous and sensitive method. In Bangladesh, late abortion occurred in dairy cattle by different types of causal agents. of them, *Brucella* spp is the major causal agent.

In this study *Brucella* spp is the first time isolated and identified in molecular level from aborted fetus of dairy cattle in Bangladesh. It is a Nobel finding in aspect of Bangladesh. The Nobel bacteria may be used as a vaccine candidate for production of vaccine against control of bacterial abortion. The desire vaccine may prevent late abortion in dairy cattle of Bangladesh and help the dairy development as well.

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# Annex-i

Table 01: Growth in *Brucella* spp. from aborted fetuses on Columbia agar/ Peptone broth base:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Growth on Columbia agar base with Brucella selective supplement | | | | Results on Gram Stain and Remarks |
|  | | | |
| Eyeball | Liver | Lung | Kidney |
| F1 (Fetus 1) | Growth after 24 hours | Growth after 72 hours | Growth after 72hours | Growth after 72 hours | Positive for *Brucella.* |
| F2 (Fetus 2) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F3 (Fetus 3) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F4 (Fetus 4) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F5(Fetus 5) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F6 (Fetus 6) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F7 (Fetus 7) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F8 (Fetus 8) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F9 (Fetus 9) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F10 (Fetus 10) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F11 (Fetus 11) | No Growth | No Growth | No Growth | No Growth | Positive for *Brucella .* |
| F12(Fetus 12) | Growth after 24 hours | Growth after 96 hours | No Growth | No Growth | Negative for *Brucella.* |
| F13(Fetus 13) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F14 (Fetus 14) | No Growth | No Growth | No Growth | No Growth | Positive for *Brucella* |
| F15 (Fetus 15) | Growth after 48 hours | Growth after 96 hours | No Growth | No Growth | Negative for *Brucella.* |
| F16 (Fetus 16) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F17 (Fetus 17) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F18 (Fetus 18) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella.* |
| F19(Fetus 19) | No Growth | No Growth No Growth | No Growth | No Growth | Negative for *Brucella* |
| F20 (Fetus 20) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F21 (Fetus 21) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F22 (Fetus 22) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F23 (Fetus 23) | No Growth | No Growth | No Growth | No Growth | Negative for *Brucella* |
| F24 (Fetus 24) | Growth | Growth | No Growth | No Growth | Positive for *Brucella* |
| F25 (Fetus 25) | Growth | Growth | Growth | No Growth | Positive for *Brucella* |

# Annex-ii

The farm that were selected for aborted fetus collection

|  |  |  |  |
| --- | --- | --- | --- |
| Commercial Dairy Cows | | | |
| Sl. No. | Name of the farm | Address of the farm | Total no. of cows (under investigation) |
| 01. | Molla Dairy farm | Patenga | 03 |
| 02. | Bhuiya Dairy farm | Baizid Bostami | 02 |
| 03. | Homeland Dairy farm | Chandgaon | 01 |
| 04. | Janata Dairy farm | Foy’s Lake, Khulshi | 01 |
| 05. | Rumy Dairy farm | Raojan | 00 |
| 06. | Paharika Farms Ltd. | Fatikchari | 02 |
| 07. | Dairy Fair | Chandgaon | 01 |
| 08 | Lisa Dairy farm | Shikolbaha | 02 |
| 09 | Madina Dairy farm | Shikolbaha | 01 |
| 10 | Musa Dairy farm | Shikolbaha | 00 |
| 11 | Hasan Dairy farm | Shikolbaha | 01 |
| 12 | Amena Dairy farm | Shikolbaha | 01 |
| 13 | Moinundin Dairy farm | Shikolbaha | 02 |
| 14 | Shazahan Dairy farm | Shikolbaha | 01 |
| 15 | Senuara Dairy farm | Anwara Thana | 01 |
| 16 | Kumar Dairy farm | Anwara Thana | 01 |
| 17 | Ibrahim Dairy farm | Anwara Thana | 00 |
| 18 | Abu Dairy farm | Anwara Thana | 00 |
| 19 | Hannah Dairy farm | Anwara Thana | 01 |
| 20 | Hafsa Dairy farm | Anwara Thana | 02 |
| 21 | Sebuara Dairy farm | Anwara Thana | 01 |
| 22 | Rahim Dairy farm | Anwara Thana | 00 |
| 23 | Delwar Dairy farm | Anwara Thana | 01 |
| 24 | Bodruddin Dairy farm | Anwara Thana | 00 |
| 25 | Habib Dairy farm | Anwara Thana | 01 |

# Annex-iii

Some snapshots of reagents and working procedure: