MOLECULAR CHARACTERIZATION OF LEPTOSPIRA HARDJO IN CATTLE

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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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This thesis is dedicated to my loving parents. Without their knowledge, wisdom, and guidance, I would not have the goals I have to strive and be the best to reach my dreams!

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List of abbreviation

Abbreviation	Elaboration
%	Percent
gm	Gram
Kg	Kilogram
ml	Milliliter
µg	Micro gram
≤	Less than or equal to
≥	Greater than or equal to
^{o}C	Degree Celsius
WHO	World Health Organization
FAO	Food and Agricultural Organization
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
EMJH	Ellinghausen-McCullough-Johnson-Harris
ELISA	Enzyme Link Immuno-Sorbent Assay
min	Minute

Abstracts

Leptospirosis is a potential threat to dairy industry responsible for early embryonic death and infertility which can lead to significant economic losses for cattle producers. A cross sectional study was conducted for molecular characterization of Leptospira Hardjo in sero-positive dairy cows by using polymerase chain reaction (PCR) technique. Organisms were isolated from urine and fetus by using Ellinghausen-McCullough-Johnson-Harris (EMJH) media and initial tracing by dark field microscopy from 45 sero-positive selected cows. Prevalence of Leptospira Hardjo in dark field microscopy was 55.55% (25 out of 45) and 32% (8 out of 25) respectively in urine and fetus samples. No band was found in PCR from urine samples (none out of 45) besides 32% (8 of 25) fetal samples were found positive for L Hardjo. Maximum cases of abortion were occurred during the second trimester of pregnancy and their difference among the gestation varies significantly ($P<0.05$). The overall results of this study expressed that Leptospira Hardjo is one of the potential causes of abortion in the dairy industry of Chittagong, Bangladesh.

Chapter-1: Introduction

Leptospirosis is caused by pathogenic spirochaetes of the genus Leptospira. The organism affects many mammalian species, including humans. Animals may become in-apparent carriers and shedding of leptospires primarily in the urine which serves as a source of infection for other animals and humans (Mayer-Scholl et al., 2014). In cattle, leptospirosis is an important cause of abortion, stillbirths, infertility, poor milk production and death; all of which cause remarkable economic loss (Ellis, 2015). However there are many etiologies of abortions are also responsible for stillbirths, mummification and weak or deformed neonates. 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.

Dairy cattle industry is one of the major sub-sectors of animal agriculture (Livestock) in Bangladesh where people commonly live in close contact with livestock. Infertility and abortion are main problems among pregnant cows and possibly due to Leptospira Hardjo. Leptospirosis is a serious zoonotic disease with important veterinary and public health impacts (Chethan-Kumar et al., 2013). Bangladesh has experiences of flooding almost every year. The geographical location, climatic conditions and rich fauna seem to be suitable for the survival of Leptospira Hardjo. The causative organisms are shed in urine and survive in surface water, streams, or moist, alkaline soil. There are more than 100 serotypes of Leptospira but only seven serotypes have been recognized in cattle (McLean et al., 2014). Serovars causing infection in cattle have also been classified into two groups: (a) those adapted to and maintained by other cattle (serovar Hardjo); and (b) incidental infection caused by strains maintained by other domestic and free living animals (Ellis, 1994). Leptospirosis is often considered as a worldwide zoonotic disease. Studies determined that rural people in Bangladesh are at high risk to leptospiral infection (Morshed et al., 1994; Kendall et al., 2010). Leptospirosis has spread from its traditional rural base to become the cause of epidemics in poor urban slum communities in developing countries (McBride et al., 2005). The incidence of leptospirosis is significantly higher in warm climate countries than in temperate regions. Leptospirosis has been greatly under reported due to non-

specificity of sign symptoms and limited availability of laboratory confirmation in endemic regions (Laras et al., 2002). Overall disease burden is underestimated as the disease has clinical features similar to many other illnesses and there is a lack of simple, rapid tests, particularly in underdeveloped countries that hampers early management (Safiullah et al., 2009).

Leptospirosis has not been yet reported in farm animal in Bangladesh, although it has been reported in neighboring countries such as India (Ratnam et al., 1987; Venkataraman et al., 1991; Himani et al., 2013) and in Pakistan (Anwar et al., 2013). In cattle, leptospirosis can produce an abortion rate of up to 30 percent when it occurs during the final third of pregnancy (Laras et al., 2002). Reliable estimates of the prevalence of serovar Hardjo infections have not been available in the U.S. because of the difficulty in establishing the diagnosis. In a study, tested urine and serum from 15 cows in each of 44 dairy herds from four different regions of the U.S. Overall, at least one infected cow was detected in 59% of the herds tested and, in most cases; serologic results indicated that the likely infecting serovar was Hardjo. When serovar Hardjo infection becomes endemic within a herd or region, it is common to have 30 to 40% of the animals infected and shedding the organisms in their urine at any one time (Bolin, 2003). Leptospires may be visualized in clinical material by dark field microscopy, immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, cerebro-spinal fluid and dialysate fluid has been used to rapidly detect the presence of leptospires and is useful in situations where laboratory resources are limited (Levett, 2001). On the other hand, molecular diagnosis such as PCR technique has been evaluated by several groups for its usefulness in the detection of leptospiral DNA from both human and animals (Budihal and Perwez, 2014).

The specific objectives of the present study were enlisted as follows:

i) To isolate and identify the Leptospira Hardjo from dairy cows of Chittagong, Bangladesh.
ii) To characterize the isolated Leptospira Hardjo at molecular level.

Chapter-2: Review of literature

2.1. History of the aspects of leptospirosis

The reported symptoms of jaundice associated with leptospirosis are date back to the 1700's (Faine et al., 1999). The acute form of leptospirosis characterised by renal failure with accompanying jaundice and nephritis, was first reported by Adolf Weil in 1886 in Germany and was later named Weil's disease (Faine et al., 1999). Clinical reports of "infectious jaundice and fever" in soldiers and sewer workers were documented but for a long time there was no knowledge of the causative agent (Faine et al., 1999). In 1914, Inada and colleagues isolated the causative agent of leptospirosis from the blood of Japanese miners with infectious jaundice and named it *Spirochaeta icterohaemorrhagiae* (Inada et al., 1916). A non-pathogenic form was also found in fresh water and named *Spirochaeta biflexa* **(**Wolbach and Binger, 1914). The importance of occupation as a risk factor and the role of rats as a source of human infection were discovered in 1917 (Ido et al., 1917) and the occurrence of leptospirosis in livestock was recognised some years later (Alston and Broom, 1958). A number of leptospiral serovars affecting humans and animals were subsequently described (Table 2.1). The list of leptospiral serovars grew as scientists realised the zoonotic potential of leptospirosis and hence more research was carried out on the disease in most parts of the world. Leptospirosis was first reported in Australia in 1933 and the diagnosis was made through histological examination of necropsy material (Morrisey, 1934; Johnson, 1951). Subsequently, several leptospiral serovars were isolated from human patients in Australia including; L. interrogans serovars Australis, Zanoni, Kremastos, Robinsoni, Broomi, Pomona, Szwajizak; L. kirschneri serovar Valbuzzi and L. weilli serovar Celledoni (Haake and Levett, 2015). Leptospira interrogans serovars Pomona and Hardjo were isolated from cattle in Australia in the early 1970s. Other serovars that have been isolated from cattle in Australia include serovar Australis (Campbell and Stallman, 1975), Zanoni (McClintock et al., 1993), Celledoni and Grippotyphosa (Abdollahpour et al., 1996). Leptospirosis is one of the most commonly reported zoonoses in Australia with farming occupations comprising the majority of cases (Slack et al., 2009).

Table 2.1: Leptospiral serovar isolated from animals and humans

Serovar	Date	Place	Host	Reference
Autumnalis	1918	Japan	Humans	(Kitamura and Hara, 1918)
Bataviae	1923	Indonesia	Rodents	(Faine et al., 1999)
Grippotyphosa	1928	Russia	Humans	(Faine et al., 1999)
Andaman A	1931	Andaman Is.	Humans	(Taylor and Goyle, 1931)
Canicola	1933	Netherlands	Dogs	(Faine et al., 1999)
Pomona	1937	Australia	Humans	(Lumley, 1937)
Australis	1937	Australia	Humans	(Clayton et al., 1937)
Hardjo	1958	USA	Cattle	(Alston and Broom, 1958)

2.2. Morphology

Leptospires are tightly coiled spirochaetes, usually measuring 10 to 20 µm, but occasionally cultures may contain longer cells. The helical amplitude is approximately 0.1 to 0.15 µm, and the wavelength is approximately 0.5 µm (Nakamura et al., 2014). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Figure 2.1). Two axial filaments with polar insertions are located in the periplasmic space (Zhao et al., 2014). Leptospires exhibit two distinct forms of movement, translational and rotational (Faine et al., 1999). Morphologically all leptospires are indistinguishable, but the morphology of individual isolates may vary with subculture in vitro and can be restored by passage in hamsters (Nakamura et al., 2014).

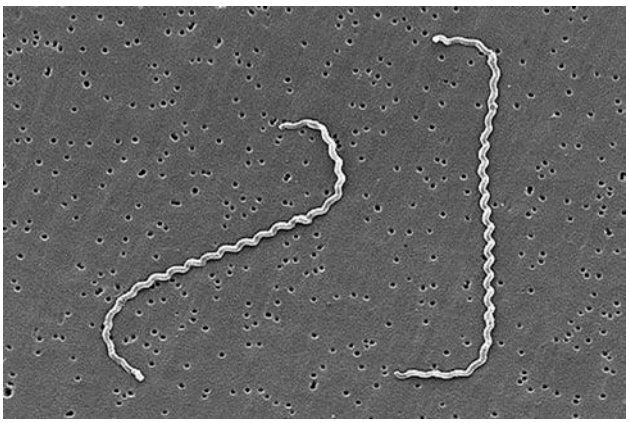


Figure 2.1: Electron micrograph of Leptospira interrogans (Wikipedia/Leptospira)

Leptospires have a distinctive double membrane structure in common with other spirochaetes, with the cytoplasmic membrane and peptidoglycan cell wall closely associated and overlain by an outer membrane (Figure 2.2) (Haake, 2000). The outer membrane appears to be fluid and contains porins that allow solute exchange between the periplasmic space and the environment. The envelope can be disorganized by salt water and desiccation. Leptospiral lipopolysaccharide has a composition similar to that of other Gram-negative bacteria, but has lower endotoxic activity (Guo et al., 2014).

Members of the genus Leptospira are obligate aerobes with an optimum growth temperature of 28 to 30^{o}C. They are unable to synthesize fatty acids and in nature only reproduce within animal hosts (Plank and Dean, 2000). They grow well in simple media enriched with vitamins (vitamins B_2 and vitamins B_{12} are growth factors), long-chain fatty acids and ammonium (Levett, 2001). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by beta oxidation salts (Levett, 2001).

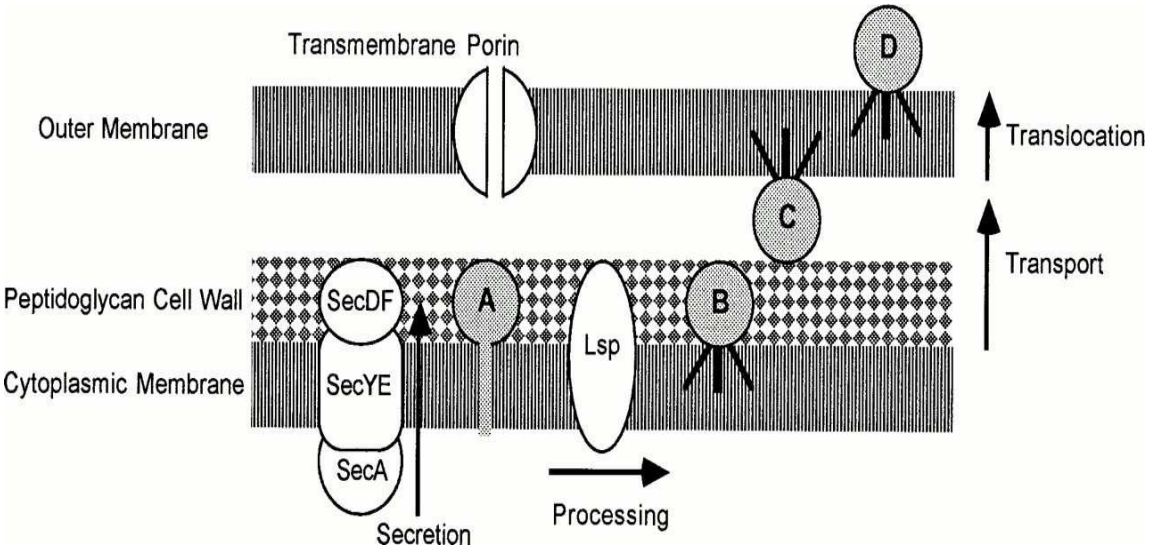


Figure 2.2: Schematic diagram of the Leptospira structure. A = prolipoprotein; B = subsurface lipoprotein in the cytoplasmic (inner) cell membrane; C = subsurface lipoprotein in the inner leaflet of the outer membrane; D = surface exposed lipoprotein (possible antigen determinant) in the outer leaflet of the outer membrane; Lsp = prolipoprotein signal peptidase (From Haake, 2000)

2.3. Genomic organization

Leptospires have a complex genome and its entire sequence of serovar Lai was established (Ren et al., 2003). The genome is large compared with the genomes of other spirochetes such as Treponema and Borrelia. This gives leptospires the ability to live in a variety of habitats such as animals or freely in the environment (Bharti et al., 2003). The genome of both the pathogenic and saprophytic species of Leptospira is approximately 5,000 kb in size (Baril and Saint Girons, 1990) although smaller estimates of 2,000 kb have been reported (Taylor et al., 1991; Bourhy et al., 2014). The genome is composed of two sections: a 4,400 kb chromosome; and a smaller 350 kb chromosome. A physical map of the Pomona chromosome of serovars subtype Kennewicki (Zuerner, 1991; Sritharan, 2012) and Icterohaemorrhagiae have been constructed. Little is known about genetic exchange among the Leptospira, although lateral transfer has been suggested (Popa et al., 2011).

Pathogenic leptospires have two sets of 16S and 23S ribosomal rRNA genes but only one 5S rRNA gene, and each rRNA gene is located far from the others on the genome (Fukunaga and Mifuchi, 1989; Baril et al., 1990; Fenner et al., 2010). Copies of several insertion-sequence (IS)-like elements (IS1500 and IS1533) coding for transposases have been identified in pathogenic leptospiral serovars but not in saprophytic species (Kalambaheti et al., 1999; Kusumoto et al., 2014). The IS1533 has a single open reading frame (ORF) and IS1500 has four ORFs (orfA-orfD) (Kusumoto et al., 2014).

Advances in molecular techniques have improved our understanding of the genus Leptospira. Analysis of 16S rRNA gene sequences indicates that leptospires are phylogenetically related to four other groups of spirochetes which include Treponema, Borrelia, Leptonema, and Brachyspira (Paster and Dewhirst, 2000; Balakrishnan et al., 2014)

2.4. Taxonomy and classification

2.4.1. Serological classification

Leptospires are spirochaetes in the order Spirochaetales and the family Leptospiraceae which includes two genera, Leptospira and Leptonema (Faine et al., 1999). Based on

serological classification, the genus Leptospira was divided into two species, *Leptospira interrogans*, comprising all pathogenic strains and *Leptospira biflexa*, containing the saprophytic strains isolated from the environment (Johnson and Faine, 1984; Rafiei et al., 2014). Leptospires are classified into over 250 serovars according to the microscopic agglutination test (MAT) that uses specific antisera to identify the distinct serovars. Serovars that are antigenically related have traditionally been grouped into serogroups (Kmety and Dikken, 1993; Bourhy et al., 2013). While serogroups have no taxonomic standing, they are useful in epidemiological studies. The serogroups of *L. interrogans* and their common serovars are shown in Table 2.2. Within some serovars, further subgroups have been identified by genomic analysis. These subgroups are types of the serovar and are serologically indistinguishable from one another (e.g. serovar Hardjo, type's hardjoprajitno and hardjobovis). It is generally considered not acceptable to refer to leptospires by the generic name followed by the serovar in italics, e.g. *Leptospira hardjo, Leptospira pomona* (Faine et al., 1999) and these should be referred to as Leptospira Hardjo and Leptospira Pomona respectively.

Table 2.2: Serogroups and serovars of clinical importance in L. interrogans (Levett, 2001)

Serogroups	Serovars
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai
Hebdomadis	Kremastos, Hebdomadis, Jules
Autumnalis	Autumnalis, Fortbragg, Bim
Pyrogenes	Pyrogenes, Zanoni
Bataviae	Bataviae
Sejroe	Hardjo, Sejroe, Saxkoebing
Grippotyphosa	Grippotyphosa
Pomona	Pomona
Canicola	Canicola, Portlandvere
Tarassovi	Tarassovi
Australis	Australis, Bratislava
Javanica	Javanica

2.4.2 Genotypic classification

The use of phenotypic characteristics to classify the species of Leptospira has recently been replaced by the use of molecular methods based on the DNA-DNA homology of the leptospiral serovars. This has given rise to a number of genomo-species, which include serovars of both *L. interrogans* (later L. interrogans sensu lato) and *L. biflexa* (later L. biflexa sensu lato). Genetic heterogeneity was initially demonstrated by (Brendle et al., 1974) and DNA hybridization studies led to the defined genomo-species of Leptospira (Ramadass et al., 1992; Ferreira et al., 2014). Unfortunately, genomo-species of Leptospira do not correspond to the previous two species (*L. Interrogans* and *L. biflexa*) and pathogenic and non-pathogenic serovars can be classified within the same species (Table 2.3). However, serogroup and serovars reliably predict the species of Leptospira therefore a combination of methods are often used. A recent study demonstrated the genetic heterogeneity within serovars which resulted in the classification of certain serovars into more than one species (Table 2.4). In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* from *L. biflexa* do not differentiate the genomo-species (Levett, 2001; Voronina et al., 2014). Therefore, a reclassification of Leptospira on genotypic grounds is taxonomically correct and provides a strong foundation for future classification. The molecular method of classification causes problems for clinical microbiologists because it is incompatible with the system of serogroups which has served clinicians and epidemiologists well for a long time. Until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic Leptospira (Levett, 2001; Bezerra da Silva et al., 2011).

Table 2.3: Genomo-species of Leptospira and distribution of serogroups (Levett, 2001)

Genomo-species	Serogroup[a]
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. fainei	Hurstbridge
L. biflexa[b]	Semaranga, Andamana
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadai	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini

a Serogroups Semaranga and Andamana contain non-pathogenic leptospires
b Non-pathogenic species

Table 2.4: Leptospiral serovars found in multiple genomo-species (Levett, 2001)

Serovar	**Genomo-species**
Bataviae	*L. interrogans, L. santarosai*
Grippotyphosa	*L. kirschneri, L. interrogans*
Hardjo	*L. borgpetersenii, L. interrogans, L. meyeri*
Pomona	*L. interrogans, L. noguchii Icterohaemorrhagiae L. interrogans, L. inadai*
Kremastos	*L. interrogans, L. santarosai*
Szwajizak	*L. interrogans, L. santarosai*
Pyrogenes	*L. interrogans, L. santarosai*

2.5. Entry

The route and mode of entry of leptospires in natural infection is not clear. Leptospires are presumed to enter directly into the bloodstream or lymphatics via a number of sites. These include the conjunctivae, the genital tract in some animals, the nasopharyngeal mucosa and possibly through the cribriform plate or the lungs following inhalation of aerosolised organisms. There is also evidence of transplacental infection at any stage of pregnancy. It is unlikely that penetration of intact skin occurs (Zhang et al., 2012).

2.6. Spread and growth

The ability of leptospires to survive and grow in tissues is a major contributor to their virulence. After entry through the open skin, leptospires are immediately exposed to the effects of non-specific factors such as pH, redox potential, electrolytes, fatty acids and other small organic molecules, some of which may be nutrients that will affect the ability of the leptospires to survive and grow (Adler and Moctezuma, 2010). Their survival in the tissues of animals is mediated by their resistance to innate immunoglobulins in tissue fluids or plasma. Leptospires do not cause an acute inflammatory response when present in tissues (Arimitsu et al., 1989) it was found

that Loa22 protein mediates a direct cytotoxic effect on NRK52E cells in a dose-dependent manner (Zhang et al., 2010).

Leptospires spread almost immediately from the site of entry via lymphatic's to the bloodstream where they circulate to all tissues. The rapid penetration of the bloodstream following intraperitoneal inoculation is faster than other bacteria (Zhang et al., 2012) and leptospires are found at first in the lungs and later in the liver and spleen (Faine, 1964). In the renal tubule leptospires migrate through the interstitial space and attach to the renal epithelial cells. Avirulent leptospires which reach the bloodstream are cleared rapidly, within several minutes of entry, by reticuloendothelial phagocytosis (Tranchimand et al., 2011).

The time taken to develop lesions is a function of the size of the inoculum (infecting dose), the rate of growth of the organisms in the host, their toxicity, and the rate of development of opsonic immunity. In natural infections the infecting dose is usually assumed to be relatively small and composed wholly of virulent organisms, which will grow uniformly without hindrance until immunity develops. Toxicity is mainly a function of the serovars of leptospires in a given host (Faine et al., 1999; Zhang et al., 2010).

2.7. Persistence and carrier sites

Leptospira affects at least 160 mammalian species and has been recovered from rats, swine, dogs, cats, raccoons, cattle, horse, dogs (even vaccinated) (Gamage et al., 2011; Koizumi and Yasutomi, 2012, Hamond et al., 2013), rats (most common), domestic and feral animals, bats, California seals and squirrels being the reservoirs (Lim, 2011; Dzupova et al., 2012; Koma et al., 2012; Muhldorfer, 2012). In humans, majority of leptospirosis occur as occupational hazards (Hartskeerl et al., 2011; Nafeev et al., 2012), prominently being encountered in tropical regions. The organism enters the body via mucous membrane via splitted milk, contaminated moist soil and vegetation, ingestion and inhalation of food and droplet aerosol of contaminated, leading to subsequent infection through conjunctivae or abraded skin while swimming or immersion in contaminated water and even can penetrate broken down skin (Wang et al., 2007; Dellagostin et al., 2011; Dzupova et al., 2012). Globally, rising incidence rates with few deaths and several outbreaks have been observed in all the continents

(Abela-Ridder et al., 2010). In India, monsoon season is favourable for the disease to occur. Waterborne and post flood outbreaks along with outbreaks in athletes and travellers participating in white water rafting have also been reported (Amilasan et al., 2012; Smith et al., 2012). Leptospires may persist and multiply in certain tissues in immunologically privileged sites following clearance from the bloodstream. These tissues include the proximal renal tubules, brain, anterior chamber of the eye and the genital tract (Faine et al., 1999; Yoo, 2010). In the kidney, growth continues exponentially, reaching a maximum concentration about 21 to 28 days after infection (Yan et al., 2010).

2.8. Toxin production

Endotoxin activity has been reported in several leptospiral serovars (Levett, 2001). Leptospiral lipopolysaccharide preparations exhibit activity in biological assays for endotoxin but at much lower potencies than in the host (Levett and Haake, 2010). The haemolysin exotoxin produced by serovars Pomona, Hardjo, Tarassovi and Ballum can cause hemolytic disease in cattle (Levett and Haake, 2010).

A protein cytotoxin has been demonstrated in strains of serovars Pomona and Copenhageni and cytotoxic activity has been detected in the plasma of infected animals (Evangelista and Coburn, 2010). In vivo, studies have shown that this toxin induces a typical histopathological effect with infiltration of macrophages and polymorphonuclear cells (Yam et al., 1970). A glyco-lipoprotein fraction with cytotoxic activity has also been recovered from serovar Copenhageni (Evangelista and Coburn, 2010).

2.9. Pathology

The primary histological lesion observed in clinical leptospirosis is damage to the endothelial membrane of small blood vessels, which is caused by leptospiral toxin. The immediate effect is to loosen the junctions between cells, allowing fluid and leptospires to migrate into extravascular spaces followed by erythrocytes wherever the damage is severe or prolonged. The secondary effects of ischemic change, anoxia and increased pressure in the tissues reinforce damage resulting in cellular functional disintegration and death of the cell (Hu et al., 2013).

Perhaps the most significant manifestation of infection with serovar Hardjo is the result of persistent infection in the reproductive tract, which can lead to infertility. The precise pathogenesis is not clearly understood but it is believed that the presence of leptospires in the epithelium of the uterus and oviducts of infected cows interferes with implantation of the embryo or other events in early pregnancy (Evangelista and Coburn, 2010). In the kidneys, interstitial nephritis is the major finding, accompanied by an intense cellular infiltration composed of neutrophils and monocytes (Evangelista and Coburn, 2010); however renal disease is not commonly reported.

2.10. Epidemiology

2.10.1. Geographic distribution:

In Australia and the Pacific Islands Leptospirosis was first recognized in Australia in 1934, among cane-workers in North Queensland with infections commonly resulting from contact with rodent urine (Emanuel et al., 1964). The agricultural workers in Queensland and other states of Australia are commonly infected with serovars Australis, Zanoni, Hardjo, Pomona, Tarassovi and Bratislava from cattle, pigs, sheep and rodents. Leptospiral serovars dominant in the tropics of Australia are Zanoni, Hardjo and Australis whilst Hardjo, Pomona, Tarassovi and Bratislava predominate in temperate regions (Smythe et al., 2000; Picardeau, 2013). Serological surveys conducted in selected Pacific Island countries showed that infections with Leptospira species are present in the region (Tubiana et al., 2013)

2.10.2. Sero- prevalence of Leptospirosis

2.10.2. A. Global Perspective.

Leptospirosis is an infectious disease caused by spirochetes of the genus Leptospira that are capable of infecting a large variety of domestic and wild mammals (Evangelista and Coburn, 2010). Initially, two species were recognized, *L. interrogens* (pathogenic) and *L. biflexa* (saprophytic). Recent DNA studies prove that at least 12 pathogenic and 4 saprophyte species exit in the nature. These species are divided in more than 250 serovars distributed in 24 serogroups (Alder and de la Peňa, 2010). However, for diagnosis and epidemiologic purposes, the antigenic classification is still used (Palaniappan et al., 2007). Leptospirosis is considered one of the major

zoonosis distributed worldwide, mainly in countries where climate is subtropical or tropical in nature since Leptospira grows best in warm and humid conditions in tropical region (Vijayayachari et al., 2008). This disease is responsible for significant economic losses to the livestock production, largely due to negative impacts on reproductive functions (abortion, embryonic death, stillbirths and infertility), decreased milk production and growth rates, as well as indirect costs associated with treatment (Ellis, 1994). However, it is complicated to estimate the real economic impact due to infected animals that often have no clinical signs of the diseases.

Knowledge of the epidemiology of bovine leptospiral infection in arid regions of Australia is limited to speculation on the importance of contact with the maintenance host and the importance of ecological niches for free – living leptospires (Black et al., 2001); these include the role of soil and low rainfall (Andrews, 1976), extreme temperatures and seasonal conditions (Durham and Paine, 1997). High prevalence of leptospiral infection in areas with higher rainfall or in areas where access to natural surface waters (Clarke, 1991) has been provided little understanding about the effects on production and economical implications of bovine leptospirosis. In South – West Queensland where 95% of tested cattle herds have shown titers to leptospiral serovars, vaccination against leptospirosis is undertaken by a limited number of properties (Clarke, 1991) including only 20% of properties in the muglalands of Queensland (O'Rourke et al., 1992). Prescott et al. (1988) studied over seroprevalence and association with abortion in cattle in Ontario, Canada by leptospirosis and was found 13.8%. The seroprevalence of leptospirosis in cattle has been reported to be 10.4% in Spain (Espi et al., 1982), 23.3% in Portugal (Collarse, 1991), 3% in Germany (Drager and Jonas, 1990), and 34.4% in Great Britain (Pritchard, 1986), and the most prevalent serovars were identified as hardjo, groppotyphosa and bratislava in these studies. Leptospirosis has been reported in India (Ratnam et al., 1987; Venkataraman et al., 1991) and in Pakistan (Ahmed, 1987). It has been postulated that Leptospirosis is maintained in nature by chronic renal infection of carrier animals. Most important reservoirs are rodents and other small mammals (e.g. mice, voles, hedgehogs), while livestock and companion animals are also significant sources of human infection. Once infected the mammals, they were excreted leotpspires intermittently or continuously throughout entire life through urination and polluted the stagnant water (Safiullah et al., 2009).

2.10.2. B. Local (Bangladesh) Perspective

Bangladesh has the flooding experiences almost every year in monsoon. The geographical location, climatic conditions and rich fauna seem to be suitable for the survival of leptospirosis. The causative organisms are shed in urine and survive in surface water, streams, or moist, alkaline soil. There are more than 100 serotypes of Leptospira but only seven serotypes have been recognized in cattle. It is a worldwide zoonotic disease. A study determined that rural people in Bangladesh are at high risk to leptospiral infection during agriculture in the field (Morshed et al., 1994).

Table 2.5: The prevalence of leptospiral infection in animals in selected Pacific Island countries **(**From Angus http://www.spc.int/rahs/Projects/zoonoses3E.htm, accessed 2014)

Country	Year reported	Seroprevalence	
		Bovine	Porcine
Micronesia	1997	No data	33%
Fiji	1994	No data	No data
Kiribati	1996	No data	3%
Palau	1999	50%	40%
Samoa	1999	40%	23%
Solomon Island	1999	83%	12%
Tonga	1996	19-45%	5-16%
Wallis & Futuna	2000	No data	28-40%

2.10. 3. Sources and modes of transmission of leptospires

Domestic and wild mammals, rodents, reptiles and amphibians are maintenance hosts for different leptospiral serovars. Rodents and cattle are considered the most important source of human infection (Smythe et al., 2000; Levett, 2001). Leptospires colonize the kidneys of carrier animals (Mayer-Scholl et al., 2014) and are shed in urine, which is the main source of environmental contamination. Estimates of the number of leptospires shed range from 10,000 to 1,000,000 organisms per milliliter of urine (Faine et al., 1999). Humans or other animals are usually infected by exposure to urine from infected animals. Other sources of transmission are contaminated

surface water (includes rivers, lakes, ponds), mud and soil (Levett, 2001; Saito et al., 2014).

The modes of transmission can be either direct or indirect. Direct transmission occurs from chronically infected animals to other susceptible animals through animal's urine (Mayer-Scholl et al., 2014). The kidneys are the site of leptospires localization and urine is the medium for transmission (Mayer-Scholl et al., 2014). In cattle and pigs there is evidence that leptospires can cross directly from the genital tract to the placenta and infect the fetus, which could have a primary or secondary role in abortion (Gamage et al., 2011). Indirect transmission occurs when animals or humans acquire infection with Leptospira from the environment through the conjunctivae, the oral mucosa, respiratory tract mucous membrane or cuts in the skin (Levett, 2001).

2.10.4. Cycle of host infection

The epidemiology of human leptospirosis reflects the ecological relationship between humans and chronically infected mammalian reservoir hosts. Humans are considered an incidental end-host from which further transmission has not been demonstrated, although individuals can excrete leptospires in their urine for several weeks (Bharti et al., 2003).

There are two natural cycles of transmission of Leptospira in Australia. A sylvatic cycle exists between rodents and marsupials and a domestic cycle involves cattle, pigs, dogs and sheep (Desvars et al., 2013).

In the sylvatic cycle, leptospirosis is accidently transmitted to farmed animals and humans from numerous species of rodents and marsupials. The principal means of spread and continuity of infection in rodents or marsupials is by direct transmission from the mother to the young. Humans can be infected through contact with an environment contaminated with rodent's urine. The most important sources for human infections are the various species of rodents with which humans live in domestic, agricultural or occupational association. Rodents closely associated with human habitation, such as the black and brown rats (*Rattus rattus* and *R. norvegicus*) and the common domestic mouse (*Mus musculus*) can act as sources of leptospires for humans, dogs and farm animals (Mayer-Scholl et al., 2014).

Maintenance hosts are animals which do not generally show signs of clinical infection but which can shed leptospires for long periods of time. Urine contaminated with Leptospira from these animals can infect humans or other non-maintenance hosts resulting in disease. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serogroups Icterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum (Levett, 2001). Urban black rats (*R. rattus*) are a maintenance host for serovars Copenhageni or Ballum whereas *R. norvegicus* harbors only serovar Copenhageni (Mayer-Scholl et al., 2014).

The domestic cycle of leptospirosis involves of cattle, pigs, sheep, buffalo, goats and dogs. Domestic animals are maintenance hosts of specific serovars; cattle usually maintain serovars Hardjo, Pomona and Grippotyphosa; pigs harbor serovars Pomona, Tarassovi or Bratislava; sheep may harbor Hardjo and Pomona; and dogs may harbor Canicola (Levett, 2001).

2.10.5. Survival of leptospires in the environment

The extent to which infection with Leptospira is transmitted depends on the survival of leptospires in the environment and on many factors, including temperature, climate, soil pH and soil moisture (Desvars et al., 2011). Moisture of the soil is important and is dependent on rainfall and water holding capacity of the soil. The survival of leptospires in soil was shown to increase as soil moisture increased (Desvars et al., 2011). Serovar Pomona was found to retain viability, pathogenicity and antigenicity for up to 74 days when recovered from soil which had a moisture content of 15.2 to 31.4% and a pH of 6.7-7.2 (Desvars et al., 2011). In an acidic soil environment, serovar Pomona was found to survive for up to 49 days (Subharat et al., 2012).

Leptospira, like other spirochaetes, are well adapted to viscous environments, in which the organisms show greater translational motility than any other bacteria. Under laboratory conditions, leptospires can remain viable for several months in water at room temperature and a pH 7.2 to 8.0 (Subharat et al., 2012). The presence of domestic sewage decreases the survival time to a matter of hours (Chan et al., 1987) but in an oxidation ditch filled with cattle slurry, viable leptospires were detectable for several weeks (Adler and Moctezuma, 2010). When soil was contaminated with urine

from infected rats or voles, leptospires survived for approximately 2 weeks (Mayer-Scholl et al., 2014). A study showed that serovar Canicola could survive in water and remain motile for 110 days at a pH of 7.2, however little is known about the mechanisms by which pathogenic leptospires persist for long periods in aqueous environments (Trueba et al., 2004).

2.11. Pathogenesis and virulence factors

The molecular mechanisms of the pathogenesis of leptospirosis remain somewhat unclear at this time. Several candidate virulence factors have been identified that might contribute to the pathogenesis of Leptospira infection and disease, including LPS (which is thought of as a general virulence factor of Gram-negative bacteria), hemolysins, outer membrane proteins (OMPs) and other surface proteins, as well as adhesion molecules.

The ability of hemolysins to lyse erythrocytes and other cell membranes makes them potential virulence factors, as demonstrated in a number of other bacterial pathogens. Several putative leptospiral hemolysins have been identified with the completion of Leptospira genome sequencing, and work is currently underway to identify their functions. Orthologs of hemolysin proteins Tly, recognized virulence factors in the spirochete Brachyspira hyodysteriae (Ter Huurne et al., 1994), are found in *L. interrogans*. Characterization of the surface- exposed TlyB and TlyC demonstrated that these leptospiral proteins did not exhibit hemolysin properties, but TlyC was found to bind extracellular matrix (ECM) components (Carvalho et al., 2009). Purified sphingomyelinase C from *L. interrogans* serovar Pomona caused the lysis of sheep erythrocytes (Berheimer et al., 1986). The sphingomyelinase C gene (sphA) was also found in another pathogenic leptospire, *L. borgpetersenii* serovar Hardjo, and the expressed protein exhibited sphingomyelinase activities (Segers et al., 1992). Hemolysin-encoding genes found in *L. interrogans* serovar Lai include a sphA homolog, sphH, coding a pore-forming protein without sphingomyelinase or phospholipase activities (Lee et al., 2002), and sph2, whose protein product induces endothelial cell and erythrocyte membrane damage (Artiushin et al., 2004). SphH and Sph2 are both expressed during human Leptospira infection (Carvalho et al., 2010) and demonstrated cytotoxic properties (Zhang et al., 2008). Another group refuted the hemolytic properties of both SphH and Sph2 (Carvalho et al., 2010); however, the

disparity in their results may be due to different folding or other properties of the recombinant proteins used for the assays. The direct role of sphingomyelinases in pathogenesis is still unclear; the absence of sphingomyelinase genes in saprophytic leptospires (Bulach et al., 2006) could suggest possible functions in virulence (Adler et al., 2010), or simply in survival in the mammalian host environment, in which certain key nutrients (e.g., iron) are limiting.

The adhesion of leptospires to host tissue components is thought of as an initial and necessary step for infection and pathogenesis. Attachment to host cells and ECM components is likely to be necessary for the ability of leptospires to penetrate, disseminate and persist in mammalian host tissues. Like other microbial pathogens, leptospires produce microbial surface components recognizing adhesive matrix molecules that might mediate colonization of host (Schwarz-Linek et al., 2004). It has been demonstrated that *L. interrogans* binds to a variety of cell lines, including fibroblasts, monocytes/macrophages, endothelial cells and kidney epithelial cells grown in vitro (Breiner et al., 2009). Although it is well-established that ECM components play a role in the interaction of the pathogen with host molecules, recent data showed that pathogenic Leptospira bind host cells more efficiently (Breiner et al., 2009). The past decade saw identification of both host cell and ECM substrates and Leptospira adhesion molecules involved in this interaction.

In silico analysis and experimental techniques (e.g., Triton X-114 fractionation, surface immunofluorescence, surface biotinylation and membrane affinity tests) can be employed to identify leptospiral surface-exposed proteins that might have potential roles in leptospire adhesion and pathogenesis (Pinne and Haake, 2009). In combination, these approaches were successful in characterizing newly identified OmpL36, OmpL37, OmpL47 and OmpL54, but the functions of these proteins remain unknown.

Outer surface proteins are good candidate leptospiral adhesions because of their surface exposed moieties. Pathogenic leptospires express a number of proteins that are at least partially surface-exposed, including LigA, LigB and LigC, which contain bacterial immunoglobulin-like domains (Matsunaga et al., 2003). Other bacterial proteins with this domain are known adhesions, such as intimin in *E. coli* (Luo et al., 2000) and invasin in *Yersinia pseudotuberculosis* (Hamburger et al., 1999). Both

LigA and LigB bind ECM components, such as elastin, tropoelastin, collagens I and IV, laminin, and especially fibronectin (Lin et al., 2009). Fibronectin-binding is modulated by calcium, and this interaction is mediated by three motifs in LigB (Lin and Chang, 2008). However, a genetic knockout of ligB did not affect virulence or colonization in acutely infected hamsters or chronically infected rats (Croda et al., 2008). This suggests the presence of other proteins that are capable of similar interactions with the host, particularly LigA, which likely has overlapping or redundant functions.

A number of *L. interrogans* proteins have been shown to bind the ECM component laminin. One of the characterized laminin-binding proteins is Lsa24/LfhH or LenA, which was also shown to bind complement factor H, factor H-related protein-1, fibrinogen and fibronectin (Verma et al., 2010). It is a member of the leptospiral endostatin-like protein (Len) family; other proteins belonging to this group (LenB, C, D, E and F) are also found to bind fibronectin (Stevenson et al., 2007). Other leptospiral proteins identified to have laminin-binding properties include Lsa21 (Atzingen et al., 2008), Lsa27 (Longhi et al., 2009), Lsa63 (Vieira et al., 2010) and a 36-kDa membrane protein (Merien et al., 2000). Both Lsa27 and Lsa63 are surface-exposed and reactive with serum samples from leptospirosis patients (Vieira et al., 2010), suggesting their possible role in host adhesion and pathogenesis, but this remains to be experimentally determined. At present, it remains unclear whether all of these proteins interact with laminin in vivo under physiologically relevant conditions, and this will be a key question to explore in the future.

The 32-kDa lipoprotein LipL32 is highly conserved in pathogenic species, absent from nonpathogens and expressed during human infection (Merien et al., 2000). This major leptospiral OMP binds collagens I, IV and V, as well as laminin (Hoke et al., 2008). LipL32 also exhibits a calcium dependent fibronectin binding activity (Tung et al., 2010). Surprisingly and disappointingly, lipL32 mutants constructed using transposon mutagenesis did not differ from wild-type pathogenic leptospires in morphology, growth rate or adherence to ECM, and were not attenuated in animal models (Murray et al., 2009). Again, the question of functional redundancy will be important but challenging to address.

Loa22, the first genetically described virulence factor in Leptospira (Ristow et al., 2007), is a lipoprotein with a peptidoglycan-binding motif similar to OmpA (Koizumi and Watanabe, 2003) and is upregulated during acute leptospire infection (Nally et al., 2007). It is highly conserved among pathogenic Leptospira, supporting a role in pathogenesis; however, the function of Loa22 is not yet known. A loa22 mutant obtained through transposon mutagenesis was avirulent in both the guinea pig and hamster models of leptospirosis. Virulence was restored upon complementation of the mutant (Ristow et al., 2007). The mutant is surface-exposed and recognized by sera obtained from human leptospirosis patients (Gamberini et al., 2005). Together, all of these results suggest that Loa22 is a good candidate for vaccine development and for investigations into the function of the protein at the molecular level.

The exposure of *L. interrogans* in vitro to temperature and osmotic conditions mimicking the host environment resulted in changes in the expression of many genes (Lo et al., 2006). In virulent strains, ligA and ligB are upregulated at physiological osmolarity (for most mammalian tissues) (Choy et al., 2007), while expression was lost when strains were culture attenuated (Matsunaga et al., 2003). Similarly, the expression of another putative virulence factor gene sph2 was induced (Lo et al., 2009), while the outer surface protein gene lipL36 was repressed (Nally et al., 2001) at physiologic osmolarity. However, most of the differentially expressed genes code for hypothetical proteins with unknown functions (Lo et al., 2006). Interestingly, more surface proteins were down-regulated at physiological temperatures (Lo et al., 2009), possibly as a mechanism by which the pathogen evades the host immune system. These DNA microarray studies demonstrated the ability to pathogenic leptospires to adapt to the shift from environmental to physiological conditions, which may facilitate invasion and establishment of disease in hosts.

2.12. Clinical features of leptospirosis

2.12.1. Humans

The majority of infections caused by leptospires are either subclinical or of very mild severity and medical attention may not be sought (Levett, 2001). This mild (anicteric) syndrome usually lasts for about a week, and coincides with the appearance of antibodies. The early symptoms resemble those of many other common febrile

illnesses including influenza, hepatitis and several acute illnesses of viral origin. Symptoms include fever, headache, myalgia, abdominal pain, conjunctival suffusion and, less often, a skin rash (Colt et al., 2014). The rash is often transient, lasting less than 24 hours. The headache is often severe and resembles the typical presentation that occurs in dengue fever, with retro-orbital pain and photophobia (Levett, 2001). Mortality is rare in anicteric leptospirosis (Gamage et al., 2014). However, in a Chinese outbreak, death was reported in 2.4% of anicteric patients, associated with massive pulmonary hemorrhages (Hu and Yan, 2014).

Icteric (acute) leptospirosis is a more severe, progressive disease characterized by generalized pains in the neck, abdomen and limbs, severe muscle pains, especially in the calf muscles, thigh and back, and pain over the tibia, affecting the gait and ability to move (Colt et al., 2014). As the disease progresses, signs of renal and hepatic failure appear leading to varying degrees of uremia and jaundice, accompanied with, or followed by, skin and mucosal hemorrhages, haemoptysis, myocarditis, progressing to death if left untreated (Colt et al., 2014). Leptospirosis is a common cause of acute renal failure, which occurs in 16 to 40% of cases (Hu and Yan, 2014).

Ocular manifestations of severe leptospirosis were identified in a large cluster of cases that occurred after flooding in India (Sakundarno et al., 2014). Anterior uveitis, either unilateral or bilateral, occurs after recovery from the acute illness in a minority of cases (Barkay and Garzozi, 1984). Uveitis is a late complication that can cause reversible or irreversible blindness in people and in horses, and may present weeks, months, or occasionally years after the acute stage of the disease (Rathinam, 2002).

2.12.2. Cattle

The most common cause of bovine leptospirosis worldwide is infection with leptospires belonging to serovar Hardjo. Two serologically indistinguishable but genetically distinct types of serovar Hardjo have been identified, Leptospira interrogans serovar Hardjo (type hardjoprajitno) and L. borgpetersenii serovar Hardjo (type hardjobovis) (Atherstone et al., 2014). Serovar Hardjo type hardjobovis is common in cattle worldwide, whilst type hardjoprajitno is found primarily in cattle in Europe (McLean et al., 2014).

The disease cycle of bovine leptospirosis is displayed in Figure 2.4. The bacteria gain entry via the eyes, mouth, nose, or through abraded skin, and enter the bloodstream. The organism multiplies for 4 to 20 days in the blood and spreads to the brain, liver, uterus, udder and kidneys, where infection is established (Atherstone et al., 2014). Serovar Hardjo generally results in asymptomatic infections or relatively mild clinical signs with an associated decreased reproductive efficiency and milk production (McLean et al., 2014). Persistent infection of the uro-genital tract is also a prominent feature of infection with serovar Hardjo. Leptospires in the proximal renal tubules of the kidney, genital tract and mammary gland appear to be protected from circulating antibodies (McLean et al., 2014). Urinary shedding of leptospires may infect other cattle in the herd and humans that come into contact with the urine.

Abortion usually occurs 6 to 12 weeks after infection in cows infected for the first time during pregnancy and most commonly in the last 4 months of gestation (de Vries et al., 2014). Abortion is likely to be accompanied by placental retention and may lead to infertility. Abortions due to infection with serovar Hardjo tend to occur sporadically as opposed to an abortion "storm" which may occur as a result of infection with serovars Pomona or Grippotyphosa (de Vries et al., 2014). Infection late in pregnancy may result in small, weakly viable calves. Diagnosis is complicated because the clinical signs are not pathognomonic for leptospirosis and the antibody titres of the dam may be low or falling at the time of abortion.

2.13. Economic importance of leptospirosis among animals

The reported prevalence values of animal infection across the world are between 2% and 46% depending on the animal species (Salina-Melendez et al., 2007). Given this wide variation in reported prevalence values and the contributions to it of factors such as climatic, animal species, time of the year, method of investigation (serovar inclusion in testing), there is not a safe way to calculate the economic impact of the infection among animals.

However, it appears that the disease is of major economic concern when it is involved in the reproductive failure of food producing animals (Bomfim and Koury, 2006). Infection of the reproductive system could result in a "storm of abortions" causing considerable economic losses from meat and milk reductions (Tooloei et al.,

2008). Furthermore, these losses appear as more significant among cattle and pigs, because these animal species are considered less resistant than small ruminants (Lilenbaum et al., 2009).

As research derived information accumulates and the disease is better understood, its economic impact could better be estimated. This needed evaluation, depends greatly on the available means to reliably investigate suspect cases, but also the importance of unapparent infection among farm animals.

2.14. Laboratory diagnosis

Diagnosis often depends on laboratory methods because clinical presentation can vary greatly. The diagnostic method selected varies depending on the samples available and the purpose of testing. Identification of the infecting serovar is of importance both epidemiologically and clinically, since this may assist in determining the source and outcome of infection. Different assays have been developed in an attempt to provide accurate diagnosis of leptospirosis, but the majority are not suitable for use in developing countries due to their requirement for maintenance of multiple strains or expensive equipment. The tests can be divided into those that detect bacteria, their antigens or genomic material and those that detect host antibody to the infecting serovars.

2.14.1. Microscopic demonstration

Leptospires may be visualized in clinical material by dark field microscopy, immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, cerebro-spinal fluid and dialysate fluid has been used to rapidly detect the presence of leptospires and is useful in situations where laboratory resources are limited; however the technique lacks sensitivity (Faine et al., 1999). The limit of detection of dark-field microscopy is approximately 10^4 leptospires/ml (Turner, 1970). Microscopic examination of blood is of value only when leptospiraemia occurs during the first few days of acute illness (Levett, 2001). In addition, false positives can occur due to misinterpretation of fibrin or protein threads, which may show Brownian motion. A high degree of operator skill

is therefore required and no information on the infecting serovars can be gained (Smith et al., 1994).

Staining methods have been applied to increase the sensitivity of direct microscopic examination. Standard stains for Leptospira have been silver impregnation techniques, strong carbol fuchsin and methylene blue, or Gram stain using a carbol fuchsin counterstain; however they are tedious and difficult to perform (Faine et al., 1999). Immunofluorescen staining is also used to demonstrate leptospires in clinical and environmental specimens such as urine, other body fluids, frozen kidneys, water and soil, because it is easy to identify leptospires and the serovars can be determined presumptively (Ellis et al., 1983; Faine et al., 1999). An immuno-histochemical method have been applied to demonstrate the expression of various specific leptospiral antigens in the tissues of experimentally infected animals and to improve the detection of leptospires in canine renal tissue (Wild et al., 2002).

2.14.2. Cultural methods

Leptospires grow in culture media containing dilute animal serum or bovine serum albumin (BSA) (Faine et al., 1999). The most widely used medium commercially available today is the Ellinghausen-McCullough-Johnson-Harris formula, known as EMJH medium. It is based on a serum-free-oleic acid-albumin medium with derivatives containing Tween-80 as the source of fatty acids and BSA as the detoxifier (Ellinghausen and McCullough, 1965). The growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil. The liquid media can be made into semi-solid and solidified media by adding agar at concentrations of 0.1 to 0.2% and 0.8 to 1.5% respectively (Faine et al., 1999).

Unfortunately, culture is slow, requires several weeks of incubation, and has low sensitivity (Bharti et al., 2003). Media should be inoculated within 24 hours of sample collection (Palmer and Zochowski, 2000). Even under optimal conditions, organisms grow slowly and cultures can be reported as negative only after a minimum of 6-8 weeks incubation, preferably as long as 4 months, before being discarded (Levett, 2003). Pure subcultures in liquid media however usually grow within 10 to 14 days. In semi-solid media, growth reaches maximum density zones beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. The pattern

of growth is related to the optimum oxygen tension and is known as a Dinger's ring or disk (Faine et al., 1999).

The visible growth of leptospiral cultures in liquid media can be seen when swirling the container against a dark background as the solution is cloudy. Fully grown cultures at cell concentrations of approximately $5x10^7$-10^8 leptospires/ml are usually turbid to the naked eye (Faine et al., 1999). However, leptospiral cultures rarely achieve the densities obtained with "conventional" bacteria, and sometimes strains which grow poorly may not attain concentrations greater than 1-$5x10^6$ leptospires/ml (Faine et al., 1999). Leptospiral cultures may be maintained by repeated subculture, or preferably by storage in semisolid agar containing haemoglobin (Faine et al., 1999). Long-term storage by lyophilization (Annear, 1974) or at -70^{o}C in glycerol (Palit et al., 1986) is also used.

Isolation of leptospires is frequently attempted from a variety of clinical specimens during acute and chronic infections. Suitable specimens including whole or clotted blood, serum, urine, cerebro-spinal fluid and tissue samples, can be inoculated into EMJH medium containing 5-fluorouracil. Cultures are incubated at 28 to 30^{o}C and are examined weekly by dark-field microscopy for up to 13 weeks or more (Faine et al., 1999; Levett, 2001). Contaminated cultures may be passed through a 0.2 µm or 0.45 µm filter before subculture into fresh medium. Identification of isolates to the serovar level is usually carried out at reference laboratories and involves time consuming cross-absorption agglutination procedures with panels of monoclonal antibodies (Smith et al., 1994; Levett, 2001).

2.14.3. Enzyme linked immuno-sorbent assay

Enzyme linked immuno-sorbent assays (ELISAs) were developed due to the deficiencies of the MAT and to produce a faster, safer and more precise assay for the detection of anti-leptospiral antibodies (Adler et al., 2010). Nevertheless, a antibody ELISA is only able to detect genus-specific antibodies and is not suitable for serogroup or serovar identification of the Leptospira (Ribotta et al., 2000). The major benefit of the ELISA is that it can be specific for the detection of IgM or IgG antibodies (Smith et al., 1994). The presence of IgM may indicate current or recent leptospirosis, but it should be noted that IgM-class antibodies can remain detectable

for several years (WHO and International Leptospiral Society, 2003). A study by Adler and co-workers (2010) using IgG and IgM ELISAs and MAT to investigate the immune response of cattle vaccinated or experimentally infected with serovar Hardjo suggested that the IgM ELISA and MAT detect different IgM antibodies by viture of the different antigen preparations used in the tests. A variety of different antigens have been tested in the ELISA. These include a carbohydrate antigen produced by phenol extraction of whole cell preparations (Thiermann and Garrett, 1983), outer sheath protein, whole lysed bacteria, formalin-fixed whole culture extract and proteinase-K-treated antigen (Ribotta et al., 2000). Comparisons of protein and carbohydrate antigens in a indirect ELISA revealed similar sensitivities and specificities for the detection of antibodies in cattle (Dhaliwal et al., 1996). Irrespective of the antigen used, the specificity of the ELISA was shown to be limited to the genus level and cross-reactivity between serovars was reported (Thiermann and Garrett, 1983; Bercovich et al., 1990).

The time post-infection that antibody may be detected varies depending on whether an IgM- or IgG-ELISA is used. Ribeiro et al. (1995) showed that anti-leptospiral IgM could be detected in the acute phase of human infection and that the ELISA was more sensitive than the MAT. In contrast, IgG-detecting ELISA may detect antibody later in the course of infection than the MAT (Gerritsen et al., 1993). The principle advantages of the ELISA are that it can be standardised, is easy to perform and is less expensive than the MAT. The disadvantages are that some ELISA systems are less specific then the MAT (Cho et al., 1989). The genus-specific antigen used in an ELISA does not give an indication of the infecting serovar and doesn't allow differentiation between vaccinated and infected cattle (Ribotta et al., 2000).

2.14.4. Polymerase chain reaction (PCR)

The PCR has been evaluated by several groups for its usefulness in the detection of leptospiral DNA from both human and animals. Although many PCRs for pathogenic Leptospira are described in the literature, only a few have been used on clinical or veterinary samples such as urine, aqueous humor during ocular complications of the disease and tissues from aborted bovine fetuses (Richtzenhain et al., 2002). The PCR has also been used to investigate the efficacy of antibiotic treatment in stopping the shedding of Leptospira by cattle (Alt et al., 2001).

PCR-based strategies for detecting specific leptospiral DNA are more useful but they require selection of specific primers to allow for amplification of the DNA. A number of primer pairs have been described based on specific gene targets (Renesto et al., 2000); including the 16S or 23S ribosomal RNA genes found in all pathogenic leptospires (Merien et al., 1992) and others have been constructed from genomic libraries (Gravekamp et al., 1993).

There is evidence that PCR assays are more sensitive than conventional diagnostic methods such as culture and dark-field microscopy, although the sensitivity of culture may vary between laboratories (Heinemann et al., 2000). The PCR may be especially useful when the immune response of the host to the infecting serovar is poor, as with the response of cattle to serovar Hardjo, or where a poor sample quality may have rendered bacteria non-viable (O'Keefe, 2002). The ability of PCR assays to identify specific serovars is limited, and authors often describe genotypic groupings of serovars rather than serovar-specific groupings (O'Keefe, 2002).

A study examined five published PCR protocols and compared them with culture and the immunofluorescence test for the ability to detect serovar Hardjo in bovine urine. The PCR was as sensitive as immunofluorescence test (90% for genus-specific detection) and had a high specificity. None of the methods were 100% sensitive (Wagenaar et al., 2000). The PCR protocols could be readily applied to routine serovar typing of clinical samples from individuals, but they may be useful for screening herds or pooled samples (O'Keefe, 2002).

Table 2.6: Selection of primers for molecular detection of *Leptospira spp.*

Reference	**Targeted gene**	**Forward**	**Reverse**
Momtaz et al., 2012	flaB	5'-TCTCACCGTTCTCTAAAGTTCAAC-3'	5'-CTGAATTCGGTTTCATATTTGCC-3'
Heinemann et al., 1999	16S rRNA	5'GGCGGCGCGTCTTAAACATG 3'	5'TCCCCCCATTGAGCAAGATT3'
Cetinkaya et al., 2000	rrs (16S)	5'-GGCGGCGCGTCTTAAACATG-3'	5'-TTCCCCCCATTGAGCAAGATT-3'
Patricia et al., 2014	lipL32	5'-CGC TGA AAT GGG AGT TCG TAT GAT T-3')	5'-CCA ACA GAT GCA ACG AAA GAT CCT TT-3
Patricia et al., 2014	ompL1	5'-TTG ATT GAA TTC TTA GAG TTC GTG TTT ATA-3')	5'-AAG GAG AAG CTT ATG ATC CGT AAC ATA AGT-3')
Radmanesh and Afshar, 2008	16S rRNA	5'-AGGGAAAAATAAGCAGCGATG TG-3'	5'-ATTCCACTCCATGTCAAGCC-3'
Biscola et al., 2011	LEP-1	5'-GGCGGCGCGTCTTAAACATG-3'	5'-TTCCCCCCATTGAGCAAGATT-3'
Moshkelani et al., 2011	16S rRNA	5'- GCG CGT CTT AAA CAT GCA AG-3'	5'- CTT AAC TGC TGC CTC CCG TAG -3'
Hamali et al., 2012	16S rRNA	5'-AGGGAAAAATAAGCAGCGATG TG-3'	5'-ATTCCACTCCATGTCAAGCC-3'
Bhure et al., 2012	LipL32	GAACCAGGCGACGGAGACTTA GTA	TGGATCAACGGGCTCACACCT
Khamesipour et al., 2014	16S rRNA	5'-GCGCGTCTTAAACATGCAAG-3'	5'-CTTAACTGCTGCCTCCCGTAG-3
Azkur et al., 2013	16S rRNA	5'-GGCGGCGCGTCTTAAACATG-3'	5'-GTCCGCCTACGCACCCTTTACG-3'

Table 2.7: Temperature set point for different stages of PCR

Denaturation	**Annealing**	**Elongation**	**Reference**
94°C for 5 min, followed by 41 cycles of 95 °C for 30 sec	50°C for 30 sec	72°C for 30 sec and a final elongation step at 72 °C for 10 min	Momtaz et al., 2012
94˚C for 5 min, followed by forty cycles of 94˚C for 15 s	56˚C for 35 s	72°C for 40 s	Patricia et al., 2014
95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min	1 min	72°C for 1 min and a ifnal extension at 72°C for 7 min	Radmanesh and Afshar, 2008
94°C for 3 min, followed by 30 cycles at 94°C for 1 min	63°C for 1 min and 30 sec	72°C for 2 min was used	Biscola et al., 2011
94°C for 5 min, followed by 30 cycles of 94°C for 1 min,	58°C for 1 min,	72°C for 1 min and a final elongation step at 72°C for 5 min,	Moshkelani et al., 2011
95°C for 5 min, followed by 35 cycles of at 95°C for 1 min,	annealing for 1 min,	extension at 72°C for 1 min and a final extension at 72°C for 7 min	Hamali et al., 2012.
95°C for 5 min followed by cycle denaturation at 95°C for 30 s;	annealing at 65°C for 30 s;	extension at 72°C for 30 s for 35 cycles and final extension at 95°C for 5 min	Bhure et al., 2012
95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min	annealing at 58°C for 1 min	72°C for 1 min. Then, a last extension at 72°C for 5 min	Khamesipour et al., 2014
95°C for 2 min; 32 cycles of 95 °C for 30 s	55°C for 30s,	and 72 °C for 30 s; and a final extension at 72 °C for 10 min	Azkur et al., 2013

Table 2.8: Advantages and disadvantages of diagnostic tests for the detection of Leptospirosis (Budihal and Perwez, 2014)

Test	Advantages	Disadvantages
Dark Field Microscopy (DFM)	Visualize *Leptospira spp.*	Lack of sensitivity and specificity. Leptospires/ml is necessary for one organism/field to be visible under DFM.
IgM ELISA	Most widely used	False positive, IgM cannot be detected in early stages of infection and can persist in blood for years.
Microscopic Agglutination Test (MAT)	Gold Standard	Less sensitive in early phase of disease. Labor intensive and complicated procedure as there is a need to maintain Leptospira strain for preparing live antigen.
Polymerase Chain Reaction (PCR)	Successful in detecting Leptospira DNA in serum and urine samples of patients	Expensive reagents, Requires large quantity of DNA. Cannot identify the infecting serovar.

2.15. Control measures in animals

An optimal program to control leptospirosis in domestic livestock should be designed to prevent clinical disease and urinary shedding of leptospires. The most effective control programs in livestock are based on the prevention of exposure, which includes measures such as isolation, herd management, antibiotic prophylaxis and vaccination.

Isolation and herd management involve strategies to prevent direct and indirect transmission of leptospires from infected adults to susceptible young stock, because active infection often persists in older animals. For this programme to be successful, successive cohorts of animals have to remain isolated to remain free from infection, until all the infected cohorts have passed through the population. In addition, adult carriers in the herd should be culled and procedures implemented to vaccinate and prophylactically treat all animals introduced onto the property (Little et al., 1992). If pigs are kept on the farm, their effluent should be contained separately and be inaccessible to cattle, and waterways should be fenced off so animals do not have direct access Tetracycline and amoxicillin are the antibiotics recommended for

the treatment of carrier animals (Faine et al., 1999). Antibiotic prophylaxis coupled with specific herd management procedures has been suggested as a method to eliminate infection with serovar Pomona in pigs.

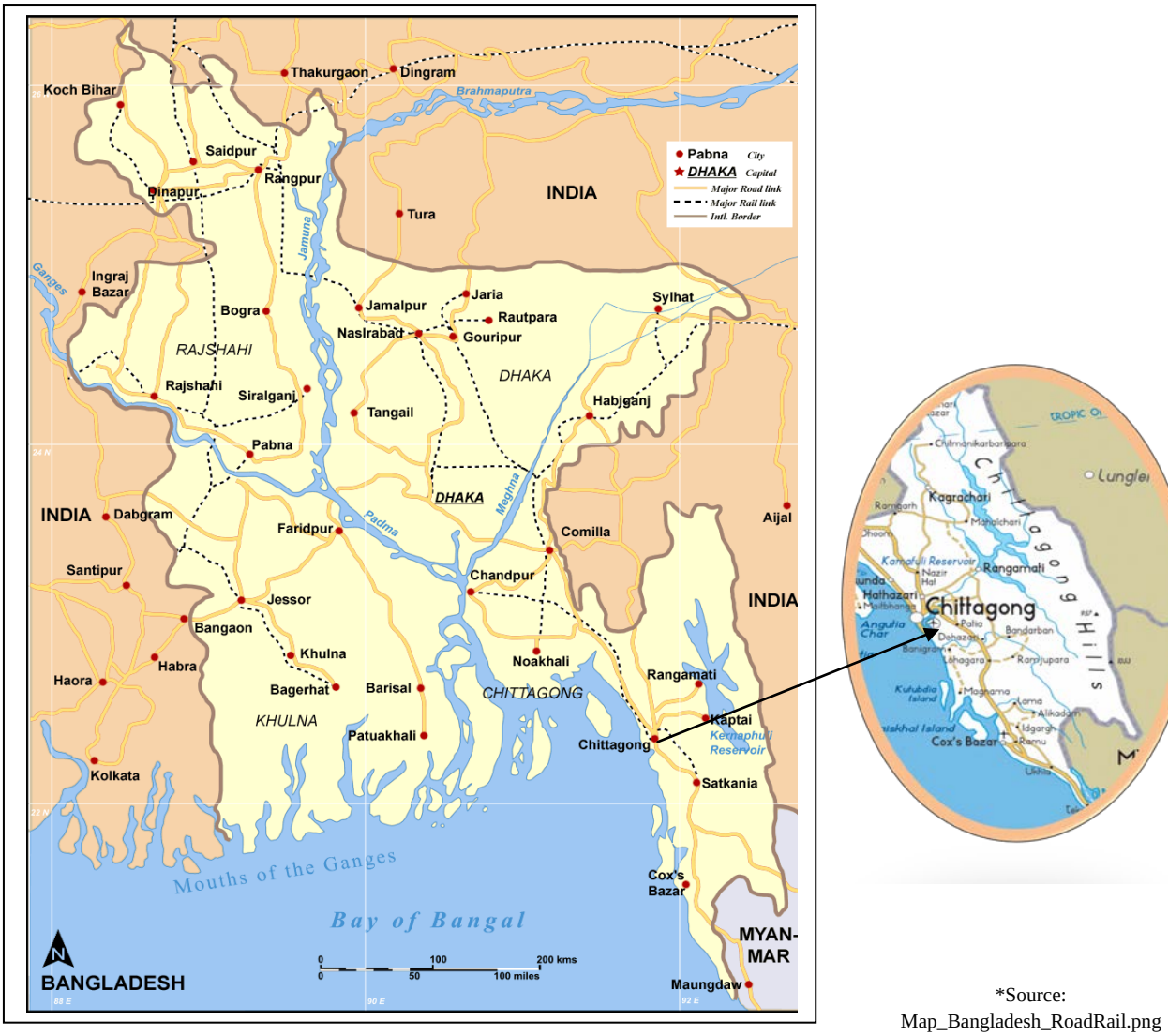
Vaccination is the most important method of preventing leptospirosis in livestock (Little et al., 1992). Depending on the degree of exposure or the level of risk, vaccinating the herd one to two times a year may be warranted (Faine et al., 1999). Calves as young as four weeks or older should initially be vaccinated, followed by a second dose four to six weeks later (Little et al., 1992). Annual revaccination maintains protective immunity but does not prevent infected animals from shedding leptospires (Faine et al., 1999).

Several field studies have shown that vaccination of cattle with infection with serovar Hardjo reduces reproductive losses and leptospiruria (Little et al., 1992). However, there have been reports that protection against infection with Hardjo in heifers has been suboptimal (Faine et al., 1999). A recently developed monovalent vaccine of Leptospira borgpetersenii serovar Hardjo has been shown to offer good protection against renal colonization and urinary shedding and has been shown to induce a cell-mediated response (Bolin and Alt, 2001). Variation in the efficacy of vaccines of serovar Hardjo is likely to be a result of a variation in vaccine composition, husbandry practices, and the pathogenicity of strains of serovar Hardjo prevalent in the region (Faine et al., 1999). Vaccines are also available for pigs and these have been shown to reduce abortion and stillbirth rates, and to reduce, but not eliminate, renal colonisation and leptospiruria (Faine et al., 1999).

Chapater-3: Materials and Methods

3.1. Study area:

Chittagong is the second most populous port city and located in coastal area of Bangladesh. The city straddles hilly terrain and faces the Bay of Bengal. It is also well known for dairy farming. Most of the dairy farm in Chittagong district is situated in the urban and peri-urban area. Keeping view the main objectives of the study the preliminary screening of farms and cows were on the basis of previous history of abortion, stillbirth, agalactia, birth of weak calves and retention of fetal membrane (>5%) and sero-prevalence of Leptospira Hardjo among the cows of Chittagong.



*Source: Map_Bangladesh_RoadRail.png

Figure 3.1: Study area map*

3.2. Selection of study population:

This research was the continuation of an ongoing project of bovine abortion caused by Leptospira Hardjo in Chittagong, Bangladesh. In the previous serological study the number of sera tested for Leptospira Hardjo was 150 while number of positive sera was 52. These 52 animals urine and aborted fetus (if) were selected for molecular diagnosis. But some of the cows were sold by the owner and animal population became 45. Finally, from this above population 45 urine samples and 23 aborted fetuses were collected from the respective cows for the preparation of inoculums.

3.3. Urine sample collection:

Urine samples were collected from the productive dairy cows of selected dairy farms under the study for detecting Leptospira Hardjo. A total of 50 ml midstream urine was collected from cows in a sterile beaker by force voiding using diuretic (Lasix^{R}). Then 15 ml of each urine sample was transferred into screw cap plastic conical tube. Then the collected urine samples were transported to lab in ice kit.

3.4. Aborted fetal sample collection:

Twenty five aborted fetuses were collected from the sero-positive dairy farms. Aborted fetuses were necropsies and internal organs such as lung, spleen, heart, liver, eyeball and abomasal contents, collected as specimens. Pieces of the internal tissues of aborted fetuses were collected with set of sterile forceps and scissors and flamed after plunging to ethanol. Each specimen was used as inoculums for culture in artificial growth medium.

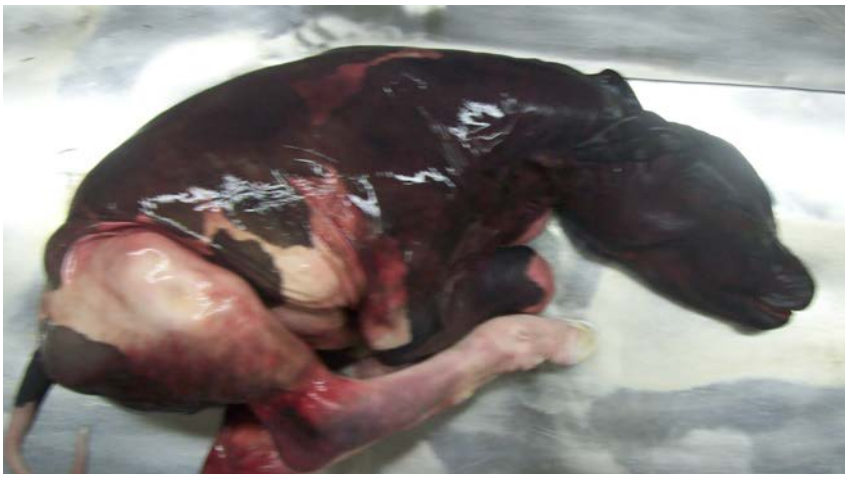


Figure 3.2: A seven months old aborted fetus collected from sero-positive dam after abortion

3.5. Media and culture protocol:

Leptospira Medium Base EMJH 2.3 gm was dissolved in 900 ml purified water and autoclaved at 121^{o}C for 15 minutes. Then aseptically Leptospira Enrichment EMJH (HIMEDIA) 100 ml was added to the above medium at room temperature and mixed thoroughly. All instruments were sterilized by autoclaving and followed strict aseptic measures. The inoculums were added to the medium and incubated at room temperature under dark place up to seven weeks. Positive growth was interpreted by enhanced cloudiness and turbidity.

3.6. Dark field microscopy technique:

About 1ml 1% formalin was added to each of the tubes and then centrifuged 2000 RPM for 10 minutes. After the centrifugation, one drop of sediment was taken on the cover slip. Then concave thick slide was applied to it. 100 fields were examined for each urine sample under Dark field microscope (DFM) (Olympus Bx50), in dry condenser.

3.7. DNA extraction protocol:

DNA of *Leptospira interrogans* was extracted by using FABGK001 (50 preps) DNA extraction kit by using the following protocol:

Initially 200µl sample 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 60^{o}C 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 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 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 FAGB 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 -20^{o}C until use.

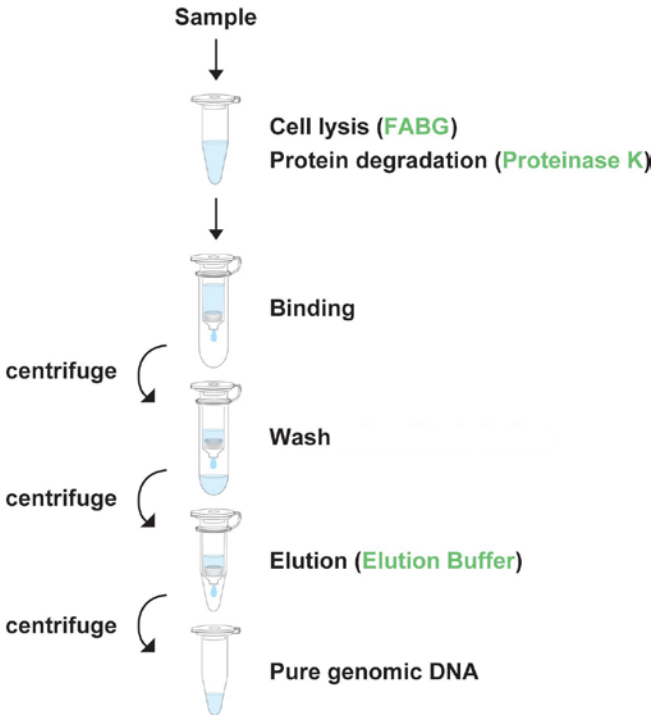


Figure 3.3: Brief procedure of DNA extraction by using DNA extraction kit

3.8. 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 *L. interrogans*, primer A, 5'-GGCGGCGCGTCTTAAACATG-3' and primer B, 5'-TTCCCCCCATTGAGCAAGATT-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 50^{o}C for 45 seconds,

extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products were detected by ethidium bromide staining after electrophoresis in 1% agarose gels. Each well received 7 ml of PCR product (green color). Tris-Boric acid-EDTA (TBE, pH 8.3) buffer was used for electrophoresis, which was carried out at 80 volts for half hour.

3.9. DNA sequencing:

From two positive samples DNA was purified for sequencing. DNA was purified from PCR product using FavorPrepTM GEL/PCR Purification Kit (FAVORGEN® BIOTECH CORP) according to the instruction of manufacturer. Briefly, with 40 µl of PCR product 5 volumes of FADF buffer was added and mixed thoroughly by vortexing. The mixture was then transferred to a FADF column and centrifuged for 30 seconds. The flow through was discarded. Again, 750 µl Wash Buffer was added to the column and centrifuged for 30 seconds. After discarding the flow through the column was centrifuged again for 3 minutes to dry and placed on to a new micro centrifuge tube. A 40 µl elution buffer was then added to the column and after standing for 2 minutes the column was centrifuged for 2 minutes to collect the eluted DNA.

3.10. DNA Sequencing

Purified PCR products were send to icddr'b, Mohakhali, Dhaka for DNA sequencing.

3.11. Phylogenetic analysis of sequences

Sequences (CVASU1 and CVASU2) were analysed using CLUSTALW software and Jalview 2.8.2 for probable clusters.

3.12. Data analysis:

Field and laboratory data obtained were entered into spread sheets of the MS Excel-2007 Program. Data were sorted and cleaned in the Excel program before exporting to STATA-11 (STATA Corp, USA). Descriptive and summary statistics were used on the results of EMJH and DFM test results. Chi-square test was used to detect the difference between the proportion of positive and negative findings on dark field microscopy and PCR. A p-value of <0.05 was considered statistically significant whereas p- value <0.01 was considered as highly significant.

Chapter- 4: Results

4.1. Dark field microscopy result for urine samples

Among 45 urine samples, 25 (55.55 %) samples were found positive for Leptospira Hardjo. both culturally and dark field microscopy test. Negative results were seen in 20 (45.45%) samples. The results are presented in Table 4.1.

Table 4.1: Results of urine samples test under Dark Field Microscopy for Leptospira Hardjo

Sl. No.	Urine samples tested from the Division of Sero – positive Dairy Farms under study	Number of urine samples tested for Leptospira Hardjo	Number of positive urine samples under DFM for *Leptospira spp.*	% of tested urine samples + ve for Leptospira Hardjo
01	Chittagong	45	25	55.55%
Overall		45	25	55.55%

4.2. Dark field microscopy result for aborted fetus samples

EMJH broth Positive samples from the 25 aborted fetuses; 8 (32%) were treated as positive for *Leptospira spp*. under dark field microscopy technique while 17 (68%) did not show the positive result (Table 4.2).

Table 4.2: Growth of Leptospira Hardjo from aborted fetus in broth medium (EMJH) N=25

Fetus Number	**Growth on Leptospira broth Medium Base (EMJH) with Leptospira Enrichment**				**Observation under Dark Field Microscope (DFM) &Remarks**
	Eyeball	**Liver**	**Lung**	**Kidney**	
F1 to F17	No Growth	No Growth	No Growth	No Growth	Negative for Leptospira Hardjo
F18 to F25	Growth after 6 weeks	Growth after 6 weeks	Growth after 6 weeks	Growth after 6 weeks	Positive for Leptospira Hardjo (Threads like structures found under DFM)

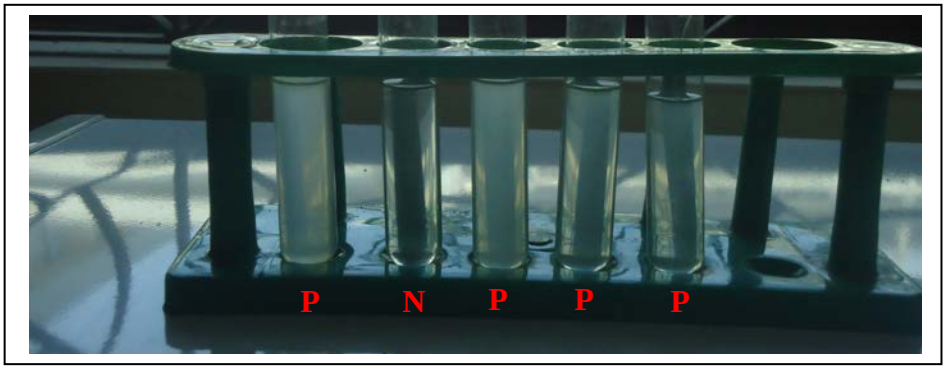


Figure 4.1: Growth of Leptospira spp. in EMJH. Positive growth (P) manifested by turbidity and cloudiness whereas a negative result (N) exhibits the transparency as like as during the time of inoculation and no change over period.

4.3. PCR results for Leptospira Hardjo from the aborted fetus

The primers used were derived from the rrs gene (16S) of *L. interrogans*. Eight samples (Table: 4.3) were produced positive band with a molecular size of 331 bp in agarose gel electrophoresis (Figure 4.2).

Table 4.3: PCR results for Leptospira Hardjo growth on EMJH

Fetus ID	Eyeball	Liver	Lung	Kidney	Pooled sample
F18 (Fetus 18)	+VE	+VE	+VE	+VE	+VE
F19 (Fetus 19)	+VE	+VE	+VE	+VE	+VE
F20 (Fetus 20)	+VE	+VE	+VE	+VE	+VE
F21 (Fetus 21)	+VE	+VE	+VE	+VE	+VE
F22 (Fetus 22)	+VE	+VE	+VE	+VE	+VE
F23 (Fetus 23)	+VE	+VE	+VE	+VE	+VE
F24 (Fetus 24)	+VE	+VE	+VE	+VE	+VE
F25 (Fetus 25)	+VE	+VE	+VE	+VE	+VE

Molecularly positive Leptospira Hardjo was found in all four organs (eyeball, liver, lung, kidney and pooled samples).

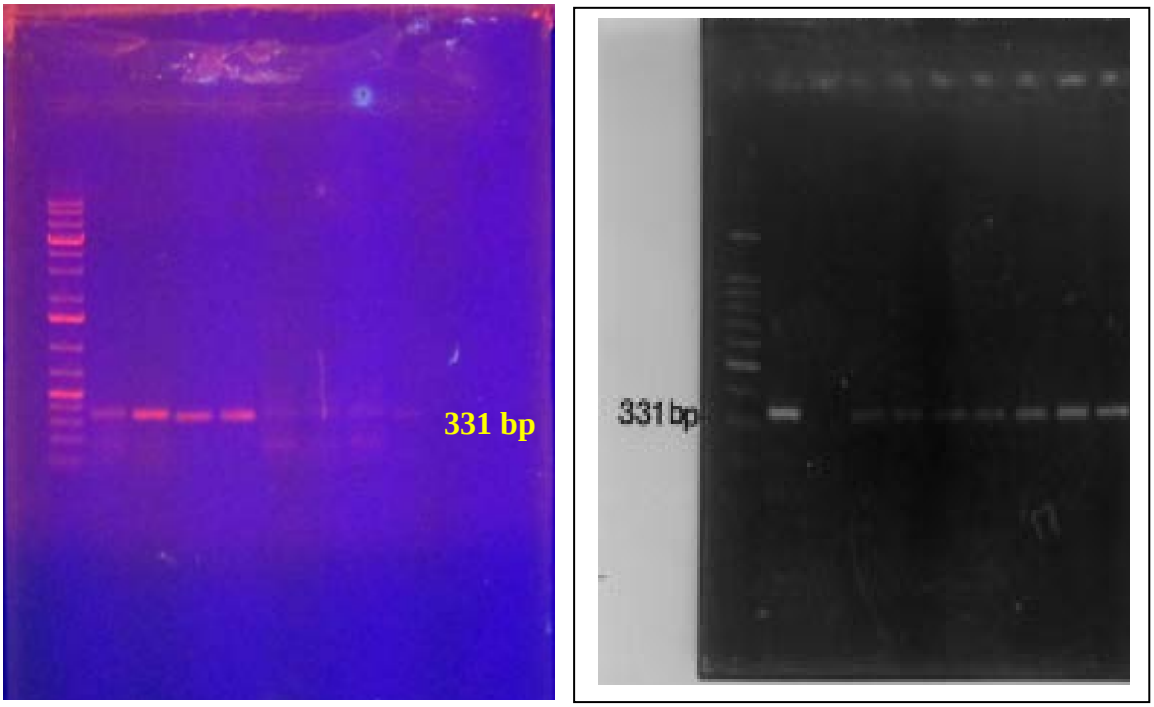


Figure 4.2: An ethidium bromide-stained agarose gel of PCR products that shows the sensitivity of the assay. DNA marker (100bp); band at 331bp (Left). Reference Cetinkaya et al. (2000) band using similar primer (Right).

Table 4.4: Period of abortion among the seropositive cows during the gestation period

Stage	No of cases	Prevalence (%)	P-value
1st trimester	3	12	0.00
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.

Table 4.5: Likelihood of occurrences of abortion in different period of gestation

Stage	No of cases	Total	P-value	Odd ratio
1st trimester	3	25	0.00	13.04
2nd trimester	16			
1st trimester	3		0.30	2.32
3rd trimester	6			
2nd trimester	16		0.00	5.63
3rd trimester	6			

The occurrences of abortion were found 13.04 times more at second trimester compared to the first trimester of pregnancy and this variation was highly significant. Besides, in relation to third trimester with first; 2.32 times more circumstances found in third trimester but the level was insignificant. Furthermore, between second and third trimester 5.63 times more abortion occurred in second trimester and the difference was also highly significant.

4.4. Identification of nucleotide bases according to chromatogram peak

After sequencing of the representative PCR product, the quality of the sequence was assessed manually for each nucleotide. Figure represents an example of partial chromatogram of sequence.

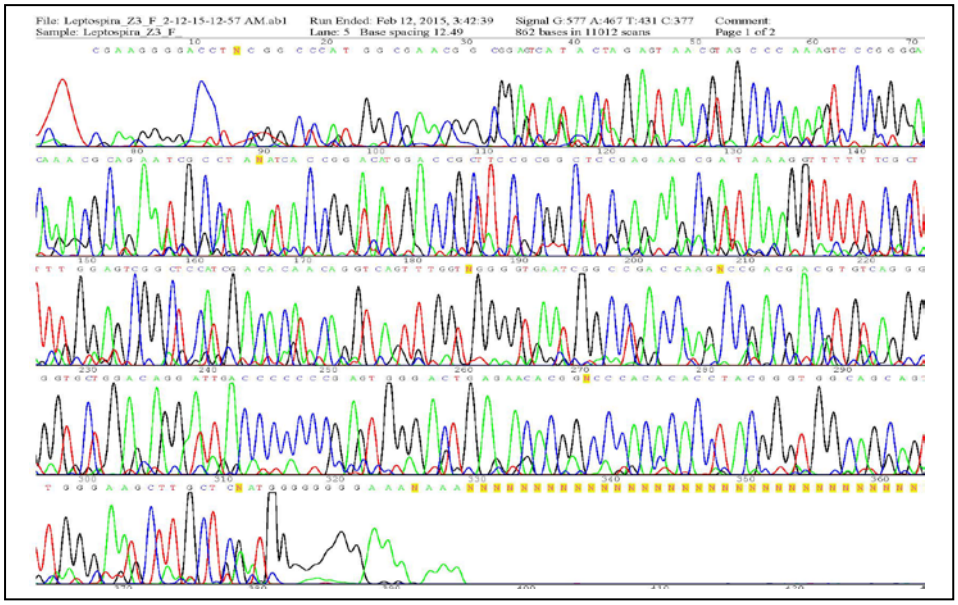


Figure 4.3: Partial chromatogram of CVASU 2

4.5. NCBI BLAST analysis

CVASU1 showed 97% nucleotide similarity with FJ154553.1 and AY996797.1. Whereas, CVASU2 showed 100% homology with KC733860.1 and JQ965147.1.

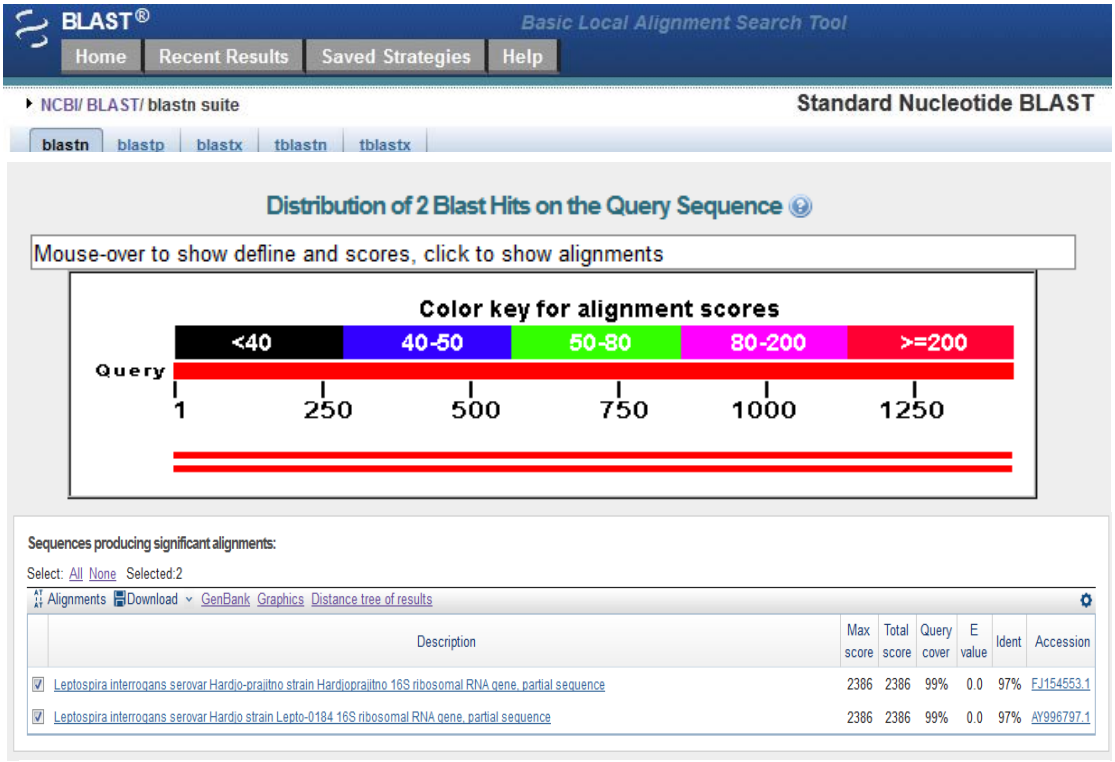


Figure 4.4: The graphic summary of 2 blast hits on the query sequence (Leptospira Hardjo CVASU1)

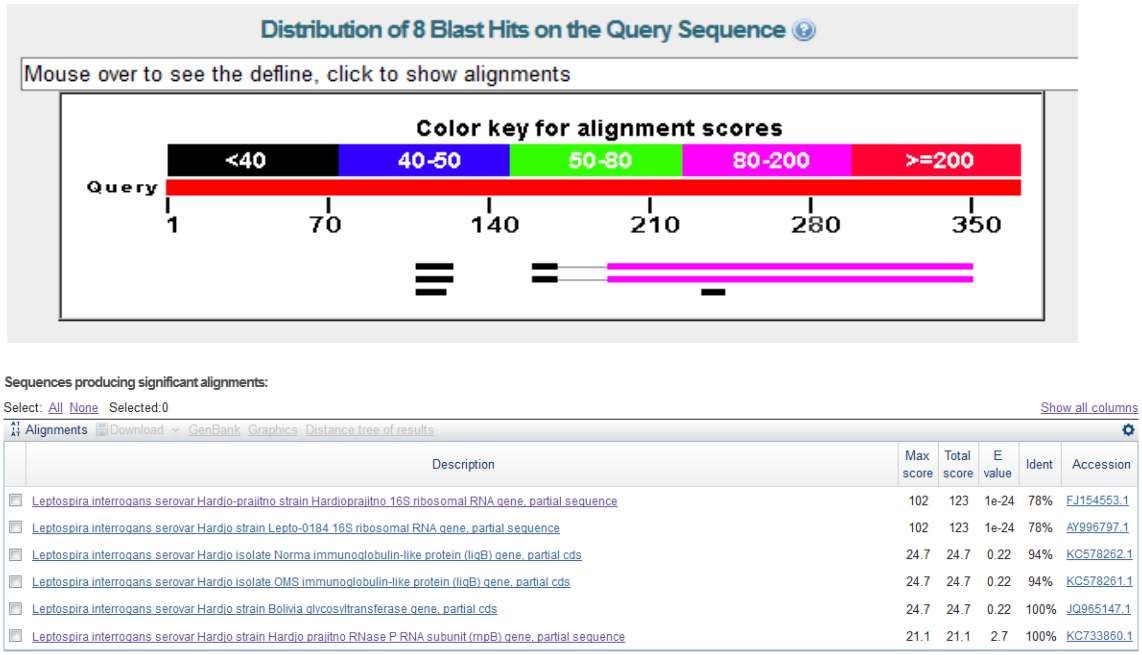


Figure 4.5: The graphic summary of 8 blast hits on the query sequence (Leptospira Hardjo CVASU 2)

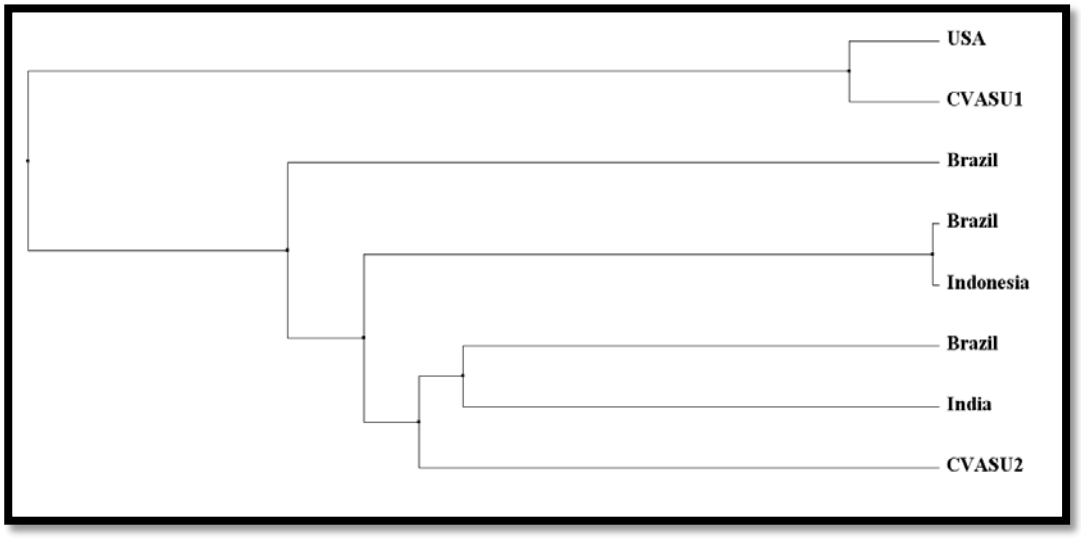


Figure 4.6: Rooted phylogram of Leptospira Hardjo CVASU1 and CVASU2 with different isolates according to the country of origin.

Chapter 5: Discussion

This study employed PCR combined with genus-specific primers in order to investigate the presence of leptospiral DNA in the urine and aborted fetus of seropositive cows at selected farms of Chittagong metropolitan, Bangladesh. This study emphasizes that Leptospira Hardjo infection is prevalent in cow in the study area. A combination of diagnostic tests was applied to the urine and fetal samples in this study to improve the sensitivity of detection. We observed more samples positive by DFM than culture and PCR combined. It is important to note that the DFM are not specific for the detection of the pathogenic Leptospira, and the possibility of false-positive results cannot be excluded (Rajeev et al., 2014).

5.1. Culture of Leptospira Hardjo in media

Leptospira spp culture is not generally attempted in diagnostic laboratories due to its laborious nature, long periods of incubation and contamination with other fast-growing bacteria. L. borgpetersenii serovar Hardjo is a very slow growing and hard to maintain species, and special media are needed to grow and maintain cultures (Rajeev et al., 2014). The diagnostic complexity due to the presence of large number of serovars and animal reservoirs emphasizes the need of culture to obtain Leptospira isolates for future epidemiologic evaluations and strategic implementation of preventive measures. L. borgpetersenii serovar Hardjo types A and B are reported in the North American cattle population (Ellis et al., 1988). Leptospira isolates belonging to serovars Pomona and Grippotyphosa have also been isolated from cattle using EMJH media these similar to our study (Tan et al., 2014).

Leptospira are typically cultivated at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, which can be supplemented with 0.21% rabbit serum to enhance growth of fastidious strains (Johnson and Harris, 1967). Growth of pathogenic Leptospira in an artificial nutrient environment such as EMJH becomes noticeable in 4-7 days; growth of saprophytic strains occurs within 2-3 days. The minimal growth temperature of pathogenic species is 13-15°C. Because the minimal growth temperature of the saprophytes is 5-10°C, the ability of Leptospira to grow at 13°C can be used to distinguish saprophytic from pathogenic Leptospira species. The

optimal pH for growth of Leptospira is 7.2-7.6 (Johnson and Harris, 1967). Room temperature was used in this study to cultivate leptospira with good growth was found up to six weeks similar to the findings of Balamurugan et al (2014). Krishna et al (2012) reported that *Leptospira spp.* isolating in culture medium, besides being difficult to perform, is dependent on factors such as: type and uniformity of culture medium, and technician's experience. In relation to the results of hamster inoculation with freshly collected semen, it was observed that the passage of material in laboratory animals could be a good alternative for isolation of *Leptospira spp.* from sample, which was also observed by Heinemann et al. (2000). Leptospira has been isolated from urine of 11 of 14 dogs in Brazil employing EMJH medium (Freitas et al., 2004). The fastidious nature of the organisms and longer generation interval and the possible contamination could be the reason for relatively low number of isolates (Thiermann, 1984). Several workers recognized the difficulty in isolating leptospires, despite the presence of leptospires in samples (Bolin et al., 2009). However, Brod et al. (2005) evaluated semen of several sires and found that it was possible to observe leptospires during a direct examination after 24 hours of cultivation in a semisolid medium. On the other hand, it was possible to detect leptospires in urine cultures from five bulls, in at least one of the collections.

5.2. Dark Field Microscopy (DFM) for early detection of Leptospira Hardjo

In the present study, DFM isolation was used for evaluating diagnosis methods for bovine leptospirosis. Though isolation of leptospires and DFM gives definitive diagnosis, the percent positivity was only 55.55% in our study whereas 68.25% detected in seropositive cases in Mannuthy by Krishna et al. (2012). Approximately 10^4 leptospires/ml of sample are necessary for visualization of one cell per field by DFM and this could be the reason for low sensitivity of DFM compared to isolation and MAT (Levett, 2001). Perhaps, serological tests are well documented for the diagnosis of leptospirosis but the regular vaccination of animals in an endemic area could be another reason for the high sero-positivity. The MAT employing live antigens is the most widely used serological test and it is the reference test against which all other serological tests are evaluated (OIE, 2008). Although MAT is considered as laborious and time consuming, relatively sensitive in comparison to DFM and isolation technique (Krishna et al., 2012).

Leptospira spp. research in freshly collected urine by direct dark field microscopy did not allow the visualization of leptospires in any of the urine samples of each animal. Direct visualization is very difficult, and a negative result does not mean absence of infection by *Leptospira spp.* (Magajevski et al., 2005). Isolation from fetus samples was also successful but 20 samples (45.45%) were found negative results perhaps they were seropositive animals. Similar frustrating results were obtained by Guimarães et al. (1987) and Heinemann et al. (1999). The failure of Leptospira isolation from the analyzed samples could be explained by the possible competition exerted by inhibitory and contaminant microorganisms present in this material (Scarcelli et al., 2001).

5.3. PCR of Urine and fetal samples:

Sample processing for PCR is critical and must be adjusted to the tissue, fluid, and species being tested. Several substances found in the various types of clinical material that may inhibit PCR; therefore positive specimens may go undetected because of false-negative results. In this experiment band was not found in PCR of urine samples although they were positive in dark field microscopy (DFM). This is supported by many references about inhibition of Taq DNA polymerase by several factors such us chelation of free magnesium ions, hemoglobin, bile salts, acidic polysaccharides from glycoproteins and extreme pH variations of urine sample (Panaccio and Lew, 2004). Greenfield and White (2013) suggested that Phenol and chloroform, often used for DNA extraction and purification are also considered to be inhibitors but it was not used in this study. As a consequence of the presence of possible inhibitors, some DNA purification steps were performed by using kits to purify DNA but result was not altered. This may be due to some bacteria can be lysed during the storage of the urine and, as a result, their DNA can be lost with the supernatant after centrifuging to concentrate the microorganisms (Paula et al., 2004).

In this study temperature for polymerase chain reaction for different steps were subjected to modify from Cetinkaya et al. (2000) even though used of same primer. These temperatures were subjected to setup by several trial and gradient PCR technique to hinder the probability of false negative results.

5.4. Leptospira in aborted fetus in relation to abortion period

Leptospirosis is likely an under-diagnosed cause of abortion in cattle and occurs worldwide. The most important serovars of *Leptospira interrogans* associated with bovine abortion are Leptospira Hardjo and L. Pomona, though rarely L. interrogans serovars icterohaemorrhagiae and grippotyphosa have been associated with bovine abortion. L. Hardjo serovars are adapted to cattle that serve as the maintenance host, whereas other serovars of Leptospira involved in bovine abortions are maintained in other domestic or wildlife species (Anderson, 2007).

Yaeger and Holler, 2007 stated that, abortion can occur 1-3 months after initial infection with L. Hardjo serovars and 1-6 weeks after infection with L. Pomona. L. Hardjo infection is associated with infertility, abortions from 4 months to term, and weak calves. In this study it was found that most abortions were occurred during the mid trimester of pregnancy that was inline of Yaeger and Holler (2007) findings and probably due to involvement of Leptospira Hardjo. Abortion due to L. Pomona usually occurs in the last trimester and significant numbers of abortion were also found during this time in this study. The herd abortion rate seldom exceeds 10% with L. Hardjo infections but can be higher with herd infections of L. Pomona. The aborted fetus is usually autolyzed. Icterus may be seen in late gestation fetuses infected with L. Pomona. Histological lesions may not be observed but in some cases, renal tubular necrosis and interstitial nephritis is present (Yaeger and Holler, 2007).

5.5. Prevalence of Leptospira Hardjo among seropositive animal's aborted fetus and urine

Prevalence of Leptospira Hardjo in aborted fetus in this study was 32%. Grooms (2006) estimates of the prevalence of Leptospira infection in a sample of US dairies and beef cow-calf operations indicated that the overall herd prevalence infection was approximately 35-50%. Paula et al (2014) reported that 30% to 40% of bovine fetuses aborted in Brazil was diagnosed multiple causes involved including *Leptospira spp.* Approximately 14.0% of fetal loss was found in the beef breeding cattle population in New Zealand of which 9% was due to *Leptospira spp* (Sanhueza et al., 2013) that was lower than our findings. In Iran 26 (28.57%) out of 91 fetus abortion was due to *Leptospira spp* and the rest on was due to *Brucella spp* and the results showed that

abortion caused by *Brucella spp.* and *Leptospira spp.* occurred mostly in first partum followed by second, third, fourth, fifth and the last partum (Dehkordi and Taghizadeh, 2012). Another study was conducted to determine the prevalence of Leptospira-induced abortions in Tabriz (north-west of Iran) dairy herds and to determine the pathogenic Leptospira serovars responsible. From May 2008 through August 2010, 16 (21.05%) of 76 submissions (fetuses and placentas) to the Large Animal Clinic of the Veterinary Faculty at the University of Tabriz were diagnosed as positive to *L. interrogans* serovars by PCR (Hamali et al., 2012).

Chapter-6: Conclusions

The hypothesis of this research was, “although bovine leptospirosis is seen in tropical countries; it could also be present in Bangladesh”. Considering the results of this study it is now revealed that Leptospira is one of the major causes of abortion in the dairy industry of Bangladesh and the prevalence is more than the brucellosis. Molecular detection of *Leptospira interrogans* was negative from the urine samples although they were positive by cultural methods and dark field microscopy technique, on the other hand positive results were found from the aborted fetus using PCR technique and the ages of the aborted fetus were significantly different.

Chapter-7: Recommendations and Future perspectives

- Veterinarians are advised to be more rigorous when approaching a farm animal with the history of abortion as *Leptospira spp.* are potential zoonoses.
- Aborted animals information and samples should be submitted to the reference laboratory (PRTC) to trace possible infection.
- Both suspected and infected animals should be isolated from the rest of the herd to ensure prompt therapeutic measures and prevention of outbreaks.
- It is the demand to develop the vaccine against field isolates Leptospira Hardjo.

Chapter-8: References

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Annex-I: List of farm and sampling details

SL	Sero-positive dairy farm name	Urine sample for culture		Total	Farm location
		Growth Positive	Growth Negative		
1	Zarif	6	0	6	Nojumiyar Hut
2	Mollah	6	6	12	Patenga
3	Sun	3	6	9	Badurtola
4	Samia	3	3	6	Halisahar
5	Nahar	6	0	6	Sitakunda
6	Madina	1	0	1	Nojumiyar Hut
7	Forhad	0	1	1	Pahartoli
8	Paharica	0	2	2	Fatikchori
9	Kazi	0	2	2	Lohagara
Total		25	20	45	

Annex-II: Ages of aborted fetus (days)

Fetus number	Age (days)	Fetus number	Age (days)
1	195	14	44
2	120	15	180
3	150	16	210
4	180	17	195
5	180	18	120
6	180	19	150
7	120	20	180
8	165	21	210
9	180	22	180
10	150	23	40
11	210	24	120
12	225	25	210
13	150		

Annex-III: Culture of samples in EMJH

Principles of the procedure

Leptospira Medium Base EMJH contains ammonium chloride, a nitrogen source, and thiamine, a growth factor. Sodium phosphate dibasic and potassium phosphate monobasic are buffering agents. Sodium chloride maintains the osmotic balance of this formula.

Leptospira Enrichment EMJH contains albumin, polysorbate 80 and additional growth factors for Leptospira.

Reagents

Leptospira Medium Base EMJH

Approximate Formula* Per Liter

Disodium Phosphate ..... 1.0 g

Monopotassium Phosphate..... 0.3 g

Sodium Chloride ..... 1.0 g

Ammonium Chloride..... 0.25 g

Thiamine ..... 0.005 g

Final pH 7.5 ± 0.2

Brief biography

Md. Ahaduzzaman has successfully passed the Secondary School Certificate (SSC) Examination in 2004 followed by Higher Secondary Certificate (HSC) Examination in 2006. He obtained his Doctor of Veterinary Medicine Degree in 2011 from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a Candidate for the degree of MS in Medicine under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. He has currently been working as an academician in Department of Medicine and Surgery since March 2014. He published eleven scientific articles in national and international journals. He has immense interest to work in small ruminant medicine.