Review of Literature
II. REVIEW OF LITERATURE

Bovine herpes virus-1 (BoHV-1), is an important pathogen of cattle which causes significant economic losses to the livestock industry worldwide. The virus has been associated with a variety of clinical disease manifestations including rhinotracheitis, vulvovaginitis, balanoposthitis, abortions, conjunctivitis, neurological disorders and generalized systemic infections (Benoit et al., 2007).

2.1 Historical Perspectives

Bovine herpes virus-1 (BoHV-1) was originally recognized as a respiratory disease of feeder cattle in the western United States during the early 1950s. The first published report on respiratory form came from Schroeder and Moys in 1954. They described an apparently new upper respiratory disease of dairy cattle that occurred in California in 1953. The disease was characterized by high fever and agalactia in addition to respiratory signs. The cause was undetermined at that time, but the disease could be transmitted with tissues and exudates from natural cases (Schroeder and Moys, 1954). The following year, Miller (1955) described a disease that was first seen in a Colorado feedlot in the fall of 1950. By 1954 it was occurring in dairy cattle and all ages of beef cattle both in feedlots and occasionally in cattle on pasture. It was known by names such as "red nose", "dust pneumonia". In the same year, the accepted name for the disease became infectious bovine rhinotracheitis (IBR). After the respiratory form of disease was characterized in the USA in the mid 1950’s, greater attention was paid to the genital form of infection as seen in female cattle. In 1958, Kendrick and co-workers described this manifestation as infectious pustular vulvovaginitis (IPV) to replace the various terms
used previously (vesicular venereal disease, vesicular vaginitis, coital vesicular exanthema, blaschenausschlag, etc.). Subsequently, the condition was widely reported in New Zealand (Webster and Manktelow, 1959); Germany (Grunder et al., 1960), Canada (Studdert et al., 1961) and South Africa (Mare and Rensburg, 1961). The first recorded outbreak of IBR in United Kingdom occurred in Oxfordshire in 1962 (Darbyshire et al., 1964). Gibbs and Rweyemamu (1977) stated that the term BoHV-1 refers to all virus isolates that are serologically related to IBR/IPV virus and it is a type species of the Herpesviridae family.

The disease was first reported in India by Mehrotra et al. (1976) who had isolated virus from cases of keratoconjunctivitis amongst crossbred calves at an organized cattle farm in Uttar Pradesh. Later, Mehrotra et al. (1979) reported the isolation and characterization of IBR/IPV virus from a case of abortion in cow.

2.2 Description of Bovine herpes virus-1

2.2.1 Virion structure and classification of the virus

The BoHV-1 genome is made of a long double-stranded linear DNA molecule arranged as a class D genome. The total size is 135.3 kilo base pairs (kbp) (Benoit et al., 2007). The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity (OIE, 2010). The virus has an icosahedral nucleocapsid (95-110 nm in diameter) consisting of 162 capsomeres (12 nm long and 11.5 nm wide with an axial hole of 3.5 nm). The nucleocapsid is surrounded
by an electron-dense zone, called the tegument and bilayer of the envelope, forming rather pleomorphic virion of 150-200 nm in diameter (Wyler et al., 1989).

The BoHV-1 is one of the eight herpesviruses isolated from naturally infected cattle. All members of the family *Herpesviridae* share common virion morphology based on icosahedral capsid symmetry. The growth characteristics and genome structure have helped in classifying it as a member of the subfamily *Alphaherpesvirinae* (Roizman et al., 1992). All BoHV-1 strains isolated hitherto belong to one single viral species, and are classified in three subtypes BoHV-1.1, BoHV-1.2a and BoHV-1.2b. The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (Edwards et al., 1990). Although most of the BoHV-1.1 strains have been isolated from respiratory tract diseases or abortion cases, the BoHV-1.2a strains are isolated from genital organ lesions (Engels et al., 1981). There is no specific rule on organ tropisms of BoHV-1.

### 2.2.2 Genome

The BoHV-1 genome is made of a long double-stranded linear DNA molecule arranged as a class D genome (Fig. 1). The total size is 135.3 kilo base pairs (kbp). The genome comprise two unique sequences, a unique long (UL) and a unique short (US). The latter is flanked by inverted internal (internal repeat, IR) and terminal (terminal repeat, TR) repeated sequences. During DNA replication, both the UL and the US regions can flip-flop relative to the other unique region, generating consequently four isomeric forms of the viral genome within the concatemeric DNA (Schynts et al., 2003).

The entire BoHV-1 genome sequence is already completed by an international cooperation project in 1995 (Leung-Tack et al., 1994; Khattar et al., 1995; Simard et al.,
1995; Vilcek et al., 1995; Schwyzer et al., 1996 and Meyer et al., 1997) (Genbank accession number AJ004801). This useful database is a composite sequence obtained from different BoHV-1 strain backgrounds. A total of 73 open reading frames (ORF) have been clearly identified. The BoHV-1 genome encloses ten genes encoding glycoproteins (Fig. 1). Among them, six are in the U₅, gK (U₅53), gC (U₅44), gB (U₅27), gH (U₅22), gM (U₅10) and gL (U₅1) and the remaining four are in the U₆, gG (U₆4), gD (U₆6), gI (U₆7) and gE (U₆8).

Bovine Herpes Virus-1 Glycoprotein B (gB) is an essential component of BoHV-1, which plays a major role in virus entry. It also represents dominant antigen that induces protective immunity in the natural host (Ros and Belak, 1999). Under selective conditions, the function of BoHV-1 gD in direct cell-to-cell spread and entry can be compensated for by mutations in other viral proteins (Liang et al., 1995 and Schroder et al., 1997).

2.2.3 Virus Replication

Bovine Herpes Virus-1 infection of permissive cells is initiated by a three step entry process. First, low affinity virus attachment with gB and/or gC to cell surface structures like heparan sulfate sugar moieties (Liang et al., 1991 and 1992; Okazaki et al., 1994 and Li et al., 1995). Secondly, stable binding of BoHV-1 gD to cellular specific receptors. An extended array of receptors have been identified as potential targets for gD interaction in different alphaherpesviruses (Campadelli -Fiume et al., 2000). Nectin-1 (a member of the immunoglobulin superfamily) is one such receptor that was demonstrated to serve as entry receptor for BoHV-1 (Geraghty et al., 1998). Lastly, fusion of the virion
Figure 1. (A) Organization of the BoHV-1 genome including two unique sequences, a long (U_l) and a short one (U_s). The latter is flanked by two repeated and inverted sequences (internal repeat, IR; terminal repeat, TR). DNA replication generates equimolar amounts of two main isomers that differ by the relative orientation of U_s and U_l segments (horizontal plain arrows); the latter is predominantly observed in a single orientation. However, low amounts of genome with inverted L segment are observed in BoHV-1 virions (horizontal hatched arrow). The location of the 10 genes encoding BoHV-1 glycoproteins is indicated by black arrowheads. The vertical black bar indicates the junction observed after genome circularization. (B) Schematic view of the two immediate early transcription units (IEtu-1 and -2) whose promoters are located in IR and TR. Activation of the promoter of IEtu1 located in IR leads to the transcription of the BICP4 and BICPO genes while the activation of the promoter of IEtu1 located in TR leads to the transcription of the BICP4 and circ genes. IEtu2 promoter activation leads to the transcription of the BICP22 gene. (C) Localization of the BoHV-1 region that is actively transcribed during latency giving rise to the Latency Related Transcript (LRT) (Benoit et al., 2007).
envelope with plasma membrane, a crucial process that requires involvement of four BoHV-1 glycoproteins: gD (Ligas et al., 1988 and Liang et al., 1995), gB (Gerdts et al., 2000) and the heterodimer formed by gH and gL (Meyer et al., 1998).

Bovine Herpes Virus-1 after entering cytosol, is transported towards nucleus pores by using dynein motor complex associated with microtubules in order to release viral DNA release. While the virus particle is transported to the nucleus, tegument proteins are shed in the cytosol of the infected cell where they might play important role at early times of virus infection because they are the first to encounter and interact with the intracellular environment. Viral Protein 8 (VP8) is the most abundant tegument protein of BoHV-1 (Carpenter et al., 1991 and Van Drunen et al., 1995). It localizes in the nucleus immediately after infection due to a nuclear localization signal (Zheng et al., 2004).

**2.2.4. Pathogenesis and dissemination**

Mucous membrane of either upper respiratory or genital tract is the most common route of entry of BoHV-1. Transmission by conjunctival route has also been reported. Direct nose to nose contact is the preferential way of transmission of BoHV-1 (Benoit et al., 2007). However, airborne transmissions by the aerosol route were demonstrated on short distances (Leuzinger et al., 2005). Genital infection requires direct contact at mating. Genital transmission also occurs through virus contaminated semen (Mars et al., 2000).

Variations detected in the gC of BoHV-1.1 and -1.2 (Rijsewijk et al., 1999 and Spilki et al., 2005) may account for tropism changes that occurred in strains causing the shift from IPV towards IBR. Once penetrated into the target epithelial cells, BoHV-1 sets
up the lytic replication cycle. The BoHV-1 cytopathic effect (CPE) is characterized by the cell ballooning and the rise of intranuclear inclusions. The cell death results both from necrosis and apoptosis processes during the BoHV-1 replication cycle. The rapid dissemination of the infection within a cattle herd occurs due to the massive virus replication at the site of entry and shedding of new progeny virus in the nasal mucus at high excretion titers. Average number of secondary cases generated by one primary case in a wholly susceptible population of defined density is designated as reproduction ratio (Ro). For BoHV-1 infection Ro was estimated to be at least seven in a dairy cattle herd (Hage et al., 1996).

Bovine Herpes Virus-1 relating to its subtype and strain causes disease in cattle with varied severity. Acute disease is caused by the destruction of BoHV-1-infected cells (Engels and Ackermann, 1996) leading to the onset of clinical signs. Binding of the virus triggers apoptosis of host cell (Lovato et al., 2003). The viral infection can cause respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal disease in cattle (Gibbs & Rweyemamu, 1977; Kahrs, 1977 and Biuk-Rudan et al., 1999) and can cause mastitis under experimental, but not in field conditions (Wellenberg, 1998).

Infections with BoHV-1 do not cause viremia in healthy mature cattle (Engels and Ackermann, 1996) and do not cause death in healthy mature cattle (Kahrs, 1977). However, BoHV-1 infections can cause foetal infection, viremia, death and abortion following genital infection of pregnant cows (Gibbs and Rweyemamu, 1977 and Kahrs,
and can cause fatal viremia in newborn calves in the absence of maternal antibodies in milk (Mechor et al., 1987).

Bovine Herpes Virus-1 spreads locally in two different ways from the mucosa. First, the viruses released in the extra-cellular medium are fully enveloped particles able to interact with the receptors of susceptible cells. Otherwise, viral particles can directly spread from an infected cell to neighboring uninfected cells. Glycoproteins gB, gD, and gH/gL are required for the cell to cell spreading, whereas gG and the heterodimer formed by gI/gE promote the direct cell to cell spread in alphaherpesvirus HSV-1 infections (Dingwell et al., 1994). Systemically the virus spreads in the host by viremia causing other clinical manifestations (Wyler et al., 1989) like abortion in pregnant cows (Miller et al., 1991a and 1991b) and fatal systemic infection in very young seronegative calves (Higgins and Edwards, 1986; Mechor et al., 1987; Bryan et al., 1994 and Kaashoek et al., 1996).

2.2.5. Latency and reactivation

One of the important characteristics of BoHV-1 is establishment of life long latency in sensory neurons of the peripheral nervous system after replication in mucosal epithelium. Bovine Herpes Virus-1 is thought to penetrate the terminus of the sensitive nerves distributed in the infected epithelium and transported along the microtubules of the axons to reach the neuron body in the nervous ganglion (Enquist et al., 1998). During latency, a latency related transcript (LRT) region is expressed in BoHV-1 leading to the inhibition of the lytic cycle and the induction of an anti-apoptotic state of the infected cells (Henderson et al., 2004).
A protein corresponding to the N-terminus of ORF2 in LRT was detected in high amounts during latency by western blotting (Hossain et al., 1995 and Jiang et al., 1998). Inhibition of apoptosis (Ciacci-Zanella et al., 1999), inhibition of the S phase entry (Schang et al., 1996) and inhibition of BICPO expression (Geiser et al., 2002) are attributed to the functions of LRT. Reactivation from latency can occur after natural stimulus exposure (Thiry et al., 1985 and 1987) or corticosteroid treatment (Sheffy and Davies, 1972) culminating in recurrent virus transmission to uninfected animals generally without clinical signs. Once reactivated in the neurons of the trigeminal ganglion, BoHV-1 initiates a new replication cycle.

2.2.6. Clinical Signs

Bovine Herpes Virus-1 most commonly exhibit subclinical infections and the severity of the infection mainly depends on the virulence of the strain, resistance factors of the host (Kaashoek et al., 1996), the age and potential concurrent bacterial infection. High fever for four to five days (peak at 41°C) post infection may be accompanied by apathy and anorexia. Dairy cows show a significant drop in milk production during that period (Hage et al., 1996 and Van Schaik et al., 1999). After two to three days of incubation, respiratory and ocular signs are observed which include red appearance of nasal mucosa, serous to mucopurulent nasal discharge and in severe cases heavy breathing at inspiration (tracheal stridor caused by necrotic debris in the tracheal lumen) and cough. Abortions have been reported as a consequence of respiratory BoHV-1 infection of a seronegative cow (Benoit et al., 2007). Abortions due to BoHV-1 are usually observed at four to eight months of gestation in case of natural infection whereas experimental inoculation causes embryonic death prior to three months of gestation.
(Chow et al., 1964; Miller and Vander, 1986). In the genital form of the disease pustules are observed on vulva and vagina in cows whereas pustules are seen on the penis of bulls. Although the infection is restricted to the genital organs, more severe infection leading to orchitis in the bull and endometritis in the cow have occasionally been reported (Gibbs and Rweyemamu 1977).

2.2.7. Immune evasion strategies

Cattle are able to set up an efficacious immune response following the primary infection with BoHV-1 allowing in most cases recovery from disease and the arrest of virus excretion. Therefore, it may be controversial to point out role of any one the several immune evasion mechanisms in the pathogenesis of BoHV-1 described hitherto. Because cattle once infected by BoHV-1 are never able to eliminate the infection and all the primary BoHV-1 infections are leading to a life-long latent infection, we can speculate that these immune evasion strategies might play a role in facilitating BoHV-1 to establish a persistent infection. Benoit et al. (2007) in their review on mechanism of BoHV-1 survival in the host have detailed following deferent mechanisms of immune evasion strategies adopted by BoHV-1.
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(Benoit et al., 2007)
2.3 Seroprevalence

2.3.1 Seroprevalence of BoHV-1 in India

The seroprevalence of Bovine herpes virus -1 has been reported in a number of Asian countries with a varying range prevalence. Samal *et al.* (1981) using indirect Haemagglutination test, reported 56.5% BoHV-1 seroprevalence in the states of Uttar Pradesh, Haryana, West Bengal, Orissa, Andhra Pradesh, Tamil Nadu and Karnataka.

Suribabu *et al.* (1984) screened serum samples for BoHV-1 antibodies by indirect Haemagglutination test and reported a seroprevalence of 64.72% in the states of Andhra Pradesh, Karnataka, Orissa, Tamil Nadu and West Bengal. Renukaradhya *et al.* (1996) found that 50.9% cattle population in the states of Andhra Pradesh, Karnataka and Tamilnadu are seropositive for BoHV-1 antibodies.

Suresh *et al.* (1999) standardised Avidin biotin ELISA for detection of BoHV-1 antibodies in serum samples. They conducted the first large scale seroprevalence of BoHV-1 in Indian covering eighteen states and union territories. They reported 38.01% of cattle in India are seropositive for BoHV-1 antibodies. The state wise BoHV-1 seroprevalence they recorded is as follows. 96.55% in Andaman and Nicobar; 37.56% in Andra Pradesh; 69.05% in Arunachal Pradesh; 13.64% in Assam; 76.74% in Bihar; 42.50% in Goa; 10% in Gujarat; 9.095 in Haryana; 12.82% in Himachal Pradesh; 95.35% in Jammu and Kashmir; 64.22% in Karnataka; 46.67% in Madyapradesh; 77.90% in Maharashtra; 51.11% in Manipur; 13.64% in Mizoram; 100% in Orissa; 23.66% in Punjab; 60.16% in Rajastan; 20.16% in Tamilnadu and 82% in the state of Uttarpradesh.
Pharande et al. (2004) in an attempt to assess the seroprevalence of BoHV-1 in western parts of the country found that 43.65% cattle in Maharashtra and 43.65% cattle in Rajasthan were seropositive by indirect ELISA.

Some of the other important reports on seroprevalence of BoHV-1 in Indian states are 18.75% in Kerala (Rajesh et al., 2003); 17.68% in Andaman and Nicobar (Jai-Sunder et al., 2005); 10.75% in Uttaranchal (Vikrant Jain et al., 2006) and 19.2% in Karnataka (Koppad et al., 2007).

In India the most recent reports on BoHV-1 seroprevalence are by Ravi (2009) who reported seroprevalence of BoHV-1 at 18.18 per cent and 12.42 per cent respectively in cows and heifers in the state of Karnataka using Avidin- Biotin ELISA kits supplied by PD_ADMAS, Bangalore. Nandi et al. (2010) recorded that 17.5% of breeding bulls in India are seropositive to BoHV-1 when tested by an indirect ELISA. Ganguly and Mukhopadhyay (2010) adopted serum neutralisation test for detecting antibodies in serum samples and they reported 38% of cattle in Westbengal state are seropositive for BoHV-1.

Avidin–Biotin indirect ELISA kits supplied by PD_ADMAS, Bangalore has been the most widely used tool for detection of BoHV-1 antibodies in India (Suresh et al., 1999; Koppad et al., 2007; Ravi, 2009 and PD_ADMAS Annual reports, 2010-11)

2.3.2 Seroprevalence of BoHV-1 in other Asian countries

The seroprevalence of BoHV-1 has been reported in a number of other countries of Asia with a varying range of prevalence. The reported seroprevalence in various
countries are 3.7% in Bali (Wiyong, 1993); 10.4% in Indonesia (Wiyong et al., 1995); 33.97% in Iran (Kargar et al., 2001); 94.5% in Korea (Choi et al., 1982); 31.3% in Syria (Giangaspero et al., 1992); 71.6% in Taiwan (Tsai-Hsiang Jung, 2001); 23.3% in Thailand (Virakul et al., 1997); 61.17% in Turkey (Okur et al., 2007).

2.3.3 Seroprevalence of BoHV-1 in African Continent

The seroprevalence of Bovine herpes virus -1 reported in various African countries are 20.5% in Algeria (Achour and Moussa, 1996); 24% in Morocco (Reynaud et al., 1995); 51.65% in Sudan (Elhassan, et al., 2006); 25.9% in Tunisia (Ghram and Minocha; 1990); 23.28% in Zambia (Mweene et al., 2003).

2.3.4 Seroprevalence of BoHV-1 in American Continent

The reported seroprevalence of Bovine herpes virus -1 in various American countries are 64.41% in Brazil (Dias et al., 2008); 37.8% in Canada (Durham and Hassard, 1990); 74.7% in Colombia (Cesar et al., 2006); 13.66% in Mexico (Córdova Izquierdo et al., 2007); 67.6% in Peru (Zechariah et al., 2002); 29% in Surinam (Corbett et al., 1989); 37% in Uruguay (Guarino et al., 2000); 3% in USA (Behymer et al., 1991); 53% in Venezuela (Obando et al., 1999).

2.3.5 Seroprevalence of BoHV-1 in European Continent

The seroprevalence of Bovine Herpes Virus -1 has been reported in a number of European countries which includes 90% in Austria (Van Oirschot et al., 1996); 35.9% in Belgium (Boelaert et al., 2000); 85.8% in Republic of Croatia (Biuk-Rudan et al., 1999); 40% in Czech Republic (Pospíšil et al., 1996); 69% in England (Paton et al.,
1998); 20 % in France (Van Oirschot, 1995); 30-50 % in Germany (Van Oirschot, 1995); 50 % in Holland (Van Oirschot, 1995); 13.5 to 15.7 % in Hungary (Tekes et al., 1999); 34.99 % and 77.5 % in Italy (Castrucci et al., 1997; Rinaldi et al., 2007); 40 % in Netherlands (Boelaert, et al., 2000); 20 to 38 % in Poland (Ackermann and Engels, 2006); 12 % in Scotland (Msolla, et al., 1981).

2.3.6 Seroprevalence of BoHV-1 with reproductive disorders

BoHV-1 infection contributes to considerable reduction in fertility rates in infected cows. Depending on gestation period and organs involved, the infection can cause early and/or late embryonic mortality, with repetition of the oestrous cycle at regular or irregular intervals and abortion. The effects on the female reproductive organs include, vulvovaginitis, endometritis, salpingitis and oopharitis, which consequently results in infertility.

The first reported case of bovine abortion due to BoHV-1 was by Brown et al. (1957) in USA, subsequently the seroprevalence of BoHV-1 in cows with previous history of abortion has been reported by a number of investigators as 9.4 % (Stubbings and Cameron 1981), 56.84 % (Sulochana et al., 1982), 40 % (Pospíšil et al., 1996), 55.4 % (Renukaradhya et al., 1996), 20 % (Rajesh et al., 2003) and 57.1 % (Koppad et al., 2007).

Repeat breeding is one of the most commonly associated reproductive problems in BoHV-1 infections. Gibbs and Rweyemamu (1977) Rajesh and co workers (2003) found that 29.7 % of BoHV-1 seropositive cattle had repeat breeding problems. Chow et
al. (1964) found that experimental inoculation of BoHV-1 virus to heifers during early pregnancy (prior to 3 months) induced embryonic death.

Urbina et al. (2004) reported that a minimum of 2.3 % of BoHV-1 seropositive cattle compulsorily developed repeat breeding problems due to endometritis in some or the other stages of their reproductive life.

There are several reports on BoHV-1 seropositive animals developing anoestrus due to necrotizing oophoritis with severe lesions on the corpus luteum (Miller et al., 1984; Miller and Van Der Maaten, 1985; Straub, 1990 and Urbina et al., 2004).

In breeding bulls which are infected by genital routes may shed BoHV-1 in their semen (under stress conditions), long after the primary infection and must be regarded as lifelong carriers. Transmission of BoHV-1 is also possible by artificial insemination; where in semen of a single infected ejaculate may be inseminated to thousands of females with clinical consequences (Smits et al., 2000). The main causes of infertility in these cases have been due to shorter oestrous cycles, metritis, necrotizing endometritis and abortions (Miller and Van Der Maaten, 1984 & 1985; Straub, 1990; Miller, 1991; Lovato et al., 1995; Smits et al., 2000 and Jain et al., 2009)

2.4 Isolation of BoHV-1

Bovine herpesvirus –1 was first reported by Reisinger and Reimann (Reisinger and Reimann, 1928) in Germany. At that time the virus had not been identified as BoHV-1 but it was found to be associated with a wide range of clinical symptoms including rhinotracheitis. In 1956, the first isolation of BoHV-1 was reported by Madin and
coworkers. Subsequently BoHV-1 was associated with abortion (Kendrick et al., 1958), infertility (Mare and Rensburg, 1961), conjunctivitis (Abinanti and Plummer, 1961) and encephalitis in calves (French, 1962).

In India, the first case of BoHV-1 was reported by Mehrotra et al. (1976) and the first isolation was by Mehrotra (1977) who isolated the virus from naso-lacrimal duct of a calf suffering from Kerato conjunctivitis at an organized cattle farm in Uttar Pradesh.

Singh et al. (1985) found intense winter as the most suitable season for collection of samples for the purpose of virus isolation from carrier animals and has stated the virus will be relatively less active during summer season.

Singh et al. (1986) in Gujarat state screened semen samples collected from 27 apparently healthy bulls and five aborted foetal contents for the presence of BoHV-1 by inoculation in MDBK cells and they were successful in getting two virus isolates one each from semen and aborted materials.

Misra et al. (1987) reported two virus isolates from uterine mucus sample and semen samples of cattle from Orissa. Rudi et al. (1992) isolated nine isolates of BoHV-1 in bovine foetal lung cells from preputial washes collected during acute phase of a balanoposthitis outbreak in bulls in Brazil.

Mehrotra et al. (1994) observed a storm of abortions in an organised cattle farm in Haryana and they attributed the abortions to BoHV-1 after the virus was isolated from aborted foetal materials.
Mohan Kumar et al. (1994) were the first to isolate BoHV-1 in Karnataka from conjunctival swabs of a cow suffering with acute conjunctivitis with typical symptoms of infectious bovine rhinotracheitis. The BoHV-1 isolate was confirmed by neutralization with convalescent serum.

Hage et al. (1996) induced BoHV-1 by injecting dexamethasone to three seropositive animals. In their study they found that samples (nasal, conunctival and vaginal swabs) collected from all the three animals between fifth and ninth day after dexamethasone treatment yielded the virus in cell culture system. They recorded symptoms only in two of the three animals and symptomless animal also yielded the virus. In their extensive and a classical study on population dynamics of BoHV-1 they defined the reproduction ratio (Ro) i.e., the average number of secondary cases generated by one primary case in a wholly susceptible population of defined density, of BoHV-1 to be at a minimum of seven.

Pharande et al. (2004) during their study on the seroprevalence and clinical epidemiology of genital form of BoHV-1 in Maharashtra, they obtained one virus isolate from a cattle with consistent symptoms of clinical repeat breeder.

Deka et al. (2005) tested 24 semen samples for BoHV-1, 12 each from sero-positive and sero-negative bulls by virus isolation method, 11 samples produced distinct CPE, characterised by rounding and clumping of cells like bunches of grapes, followed by degeneration and detachment of the MDBK cell monolayer in about 72 Hrs to 96 Hrs after inoculation. The titre of the field isolates varied from log10^{5.24} to log10^{6.24} TCID_{50}/ml. All the virus isolates were completely neutralised by BoHV-1 antiserum and
hence confirmed as BoHV-1. The virus was isolated from 5 of the 12 sero-positive bulls (41.67 %) and 6 of the 12 sero-negative bulls (50 %). The isolates were confirmed by a method of virus neutralisation by standard Reed and Muench method. They found that all the isolates had a neutralisation index of more than 1.5.

Cardenas et al. (2006) tested 49 aborted foetal samples collected in dairy herds with high abortion rates and they found that 29 samples (59 %) yielded BoHV-1 virus with characteristic cytopathic changes in cell culture.

Mahmoud et al. (2009) during their investigations on Infectious Bovine Rhinotracheitis outbreaks obtained 23 BoHV-1 virus isolates from 191 vaginal, nasal and ocular swabs in Egyptian cattle and buffaloes.

OIE (2010) reports that isolation of BoHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive.

Saha et al. (2010) attempted isolation of BoHV-1 from nasal swabs, aborted foetuses and vaginal swab samples in MDBK cells to find out the prevalent strain in the state of West Bengal. They reported one isolate from 65 nasal swabs whereas no virus was isolated from aborted foetuses and vaginal swabs. The isolate was confirmed by neutralisation with BoHV-1 hyper immune serum. They typed the virus isolate as BoHV-
1.2. The isolates were confirmed by using monoclonal antibodies at OIE referral laboratory for BoHV-1 at Germany.

Ranganatha (2011) reported isolation of three BoHV-1 isolates in MDBK cells from forty samples collected in cattle showing respiratory distress, conjunctivitis form of the disease in Karnataka state. Virus Neutralization test (VNT) was carried out for the confirmation of field isolates using BoHV-1 hyper immune serum.

Various cell cultures have been advocated for virus isolation viz., Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine foetal lung, turbinate or trachea and established bovine cell lines (OIE, 2010). However, in most isolation studies, Madin Darby Bovine Kidney (MDBK) cell line has been the most commonly used cell line for isolation of BoHV-1 in cell culture indicator system (Mohan Kumar et al., 1994; Deka et al., 2005; Saha et al., 2010 and Ranganatha, 2011).

2.5 Detection of BoHV-1 by Molecular Methods

Molecular method for the detection of the virus in clinical samples has been the latest trend. Molecular methods include Nucleic acid hybridization and different versions of polymerase chain reaction. These techniques have become ideal diagnostic tools due to their rapidity, better sensitivity and specificity.

Several hybridization methods such as dot-blot hybridization (Vilcek et al., 1993a and 1993b), in situ hybridization and Southern blot hybridization with radio isotope labelled probes (Kibenge et al., 1994 and Xia et al., 1995) have been applied for the detection of BoHV-1 in nasal swabs and semen. Polymerase chain reaction with Southern
blot hybridization has been developed as a diagnostic tool in which 0.01 TCID50/100μl of BoHV-1 could be detected in 1:20 diluted bovine semen (Kibenge et al., 1994 and Xia et al., 1995).

### 2.5.1 Polymerase Chain Reaction

After the discovery of PCR by Kary Mullis in the year 1985, amplification of target DNA is achieved by the use of synthetic oligonucleotides that correspond to sequences within the target and use of a thermostable DNA polymerase. The reaction is assayed for the specific product in agarose gel with ethidium bromide staining or by hybridization with a cloned probe or oligonucleotide probe, or by the digestion with a restriction enzyme (Mullis, 1990).

Polymerase chain reaction is increasingly used in routine diagnostic submissions. It has the primary advantages over virus isolation of being more sensitive, rapid and it can be performed in 1 or 2 days. It is also possible to detect episomal DNA of non-replicating virus in sensory ganglia, such as the trigeminal ganglion in the latent phase of infection (Engelenburg et al., 1993).

The PCR method has been applied for the direct detection of BoHV-1 in clinical samples. The detection of BoHV-1 gB, gC, gD and thymidine kinase (tk) DNA by PCR has been described (Wiedmann et al., 1993; Kibenge et al., 1994 and Vilcek et al., 1994). The nested PCR was developed for the detection of BoHV-1 in bovine semen (Masri et al., 1996).
Engelenburg et al. (1993) reported that PCR assay could detect BoHV-1 DNA in semen at 0.25-2.5 TCID_{50}. A highly sensitive detection of BoHV-1 in semen was facilitated by purifying BoHV-1 DNA from semen. Three to five molecules of BoHV-1 DNA in 50 μl of bovine semen could be detected by PCR.

The amplification of the 468 bp fragment of the BoHV-1 genome by PCR using gI gene is described by Vilcek et al. (1993a). For successful amplification, the initial denaturation of the DNA sample at 100°C for eight min was carried out prior to the cycling at 95°C for 1 min, 56°C for 1 min and 73°C for 1 min. The presence of glycerol markedly enhanced the PCR sensitivity.

Wiedmann et al. (1993) developed a nested PCR targeting a portion of the gD gene for the detection of BoHV-1 in artificially inoculated samples of extended and raw semen. The sensitivity of this assay when tested on a supernatant from a BoHV-1 cell culture was approximately 4.5 TCID_{50}. In combination with nested PCR and reverse dot blot, this method allowed the detection of 5 x 10^3 TCID per 0.5 ml of semen.

Kibenge et al. (1994) and Xia et al. (1995) have established a PCR protocol utilizing primers in the tk gene. The PCR product was used as a DNA probe in dot-blot and Southern blot hybridizations. As low as 0.01 TCID_{50}/100μl of BoHV-1 could be detected in 1:20 diluted bovine semen by using this method.

Engelenburg et al. (1995) showed that PCR could detect positive semen samples for BoHV-1, five times more than the virus isolation.
Vilcek et al. (1995) developed a PCR assay with primers selected from the gI gene and flanking a 468 bp DNA fragment. Out of 27 samples (nasal swabs, lung, lymph nodes and tracheal mucosa) collected from 16 different outbreaks in Scotland, 18 were found positive by PCR and 13 were by virus isolation. The isolated DNA from some of the samples had to be diluted by a factor 50-100 to obtain a positive PCR result.

Xia et al. (1995) reported that PCR with Southern blot hybridization was the most sensitive method and could detect BoHV-1 in semen of artificially infected bulls for a longer period than virus isolation.

Yason et al. (1995) used a gene releaser to extract DNA and found that the sample prepared by the gene releaser showed a 100-fold increase in sensitivity compared with standard DNA extraction and modified proteinase K digestion.

Gee De et al. (1996) detected BoHV-1 DNA in 23 out of 100 nasal swabs using PCR after an outbreak in Netherlands. Using the PCR, BoHV-1 could be detected only in a limited number of semen samples over a period of two months before or two months after the outbreak. Also, not all animals that shed BoHV-1 from the nose harboured detectable BoHV-1 in the semen. The virus was detected in the semen of one bull, approximately six weeks before seroconversion. The PCR was used as a means of quality control of fresh semen from bulls that were seropositive for BoHV-1.

Sreenivasa et al. (1996) designed a pair of oligomers of 20 and 23 bp for amplifying a 381 bp sequence from glycoprotein IV gene of BoHV-1. The primer pairs were used for amplifying genomic DNA of BoHV-1 directly from cell culture fluids
under different experimental conditions such as, untreated cell culture fluid, thermal
denaturation and proteinase K treatment in presence of detergent. The results revealed
that direct thermal denaturation of cell culture fluid was sufficient to detect the virus by
PCR.

Wagter et al. (1996) developed and evaluated a PCR assay for the detection of
BoHV-1 DNA in selectively digested whole bovine semen using primers and probes
based on the nucleotide sequence of the gD gene. They used non-extended semen
samples from experimentally infected bulls to compare this assay with virus isolation.
Out of 162 ejaculates, 51 were found positive by virus isolation, whereas PCR detected
BoHV-1 DNA in 73 samples.

Kataria et al. (1997) applied PCR to detect BoHV-1 in semen samples. They
found that PCR was found to be $10^6$ times more sensitive than dot blot hybridization
method in detecting viral genome and the technique detected up to 0.01TCID$_{50}$ of BoHV-
1 in semen.

Fuchs et al. (1999) detected BoHV-1 in whole-blood samples derived from
naturally infected cattle. Sensitive PCR assays specific for gB, gC and gE of BoHV-1
allowed the detection of one BoHV-1 DNA copy in $10^5$ to $10^7$ peripheral blood
leukocytes.

Moore et al. (2000) developed PCR assay based on the selected amplification of a
portion of the viral tk gene to detect both BoHV-1.1 and BoHV-1.2 subtypes. One
hundred and five diagnostic submissions, including tissues, nasal secretions and nasal
swabs were taken from cattle with respiratory disease and tested using the routine methods of virus isolation and the FAT and the results were compared with those obtained by PCR. The PCR assay detected BoHV-1 DNA in all samples that were positive by virus isolation. BoHV-1 DNA was also detectable by PCR in raw and extended semen samples at a sensitivity of 1 TCID$_{50}$ per 50µl. The PCR assay was found more sensitive and independent of sample quality than either virus isolation or FAT and it was also found faster than virus isolation. The sample preparation method was simple involving only a few steps.

Rai et al. (2002) isolated DNA from Indian isolate of BoHV-1 and amplified 520 bp sequence of glycoprotein C gene by PCR and then cloned PCR product with plasmid vector for use as probe for diagnosis of the disease.

Deka et al. (2005) screened 51 sera samples from apparently healthy breeding bulls for BoHV-1 A-B ELISA assay, revealing a seroprevalence rate of 45.09 %. Out of 24 semen samples with 12 each from seropositive and seronegative bulls, the PCR detected BoHV-1 in 50 % and 66.67 % of semen samples of bulls from seropositive and seronegative groups, respectively.

Rola et al. (2005) investigated the cause of respiratory disease outbreak by collecting 25 sera, 25 nasal swabs and two tissue samples in dairy cattle of Puawy by means of virus isolation and PCR assays. They found that 24 serum samples were positive for IBR antibody while virus was isolated from only one nasal swab and one tissue sample. PCR with external primers had detected the presence of BoHV-1 in eleven nasal swabs and one tissue sample.
Gupta et al. (2006) established a rapid and sensitive PCR based assay for BoHV-1 in semen using primers designed from gC gene utilizing glass milk for sample preparation.

Jain et al. (2009) screened 101 semen samples for the presence of viral genome by employing gB and gC specific PCR. In their extensive study in comparing the gB and gC gene based primers, they found that gB gene was the most conserved region and hence the best gene to be targeted for disease diagnosis.

Campos et al. (2009) developed primers targeting gC gene for the identification of BoHV-1 and also developed a PCR for differentiating BoHV-1 and BoHV-5 simultaneously and separately.

Bandyopadhyay et al. (2010) amplified glycoprotein B and E (gB and gE) genes of BoHV-1 using two sets of primers in samples collected from Yak.

Maidana et al. (2010) developed a differential PCR assay for differentiating BoHV-1 and BoHV-5 targeting a conserved region of gC gene.

Lojkic et al. (2011) used PCR assays specific for gB and gC for specific detection of BoHV-1. All nasal swab samples and a lung sample were positive with both sets of primers. Three gC specific products (one lung sample and two swab samples from farms B and C, respectively) were sequenced and 400-nt fragment of BoHV-1 gC encoding gene was analyzed.
Ranganatha (2011) designed a set of gC gene based primers targeting amplification of 571 bp genome of BoHV-1 and found that PCR using the said primers was five times better test than virus isolation in detecting the virus in respiratory swab materials.

Manojkumar et al. (2011) described a PCR for detection of BoHV-1 in semen samples using primers targeting the conserved regions gD genes of the virus. They opined that usage of sodium dodecyl sulphate (SDS) and Proteinase K removes PCR inhibitors and improves the efficiency of BoHV-1 detection in semen samples.

Cordoso et al. (2012) estimated the diagnostic accuracy of glycoprotein gene and US9 gene based PCR techniques in detection of Bovine herpes virus – 1.3 (BoHV-5) in decomposed brain tissues in Brazilian slaughter houses. They found that US9 gene based PCR to be more sensitive and specific in detection of the virus, however they have reported that due to nonspecificities of conventional PCR based methods, the PCR positives have to be confirmed by virus isolation and/or by genome sequencing.

2.5.2 Cloning, Sequencing and Phylogenetic analysis

Fitzpatrick et al. (1989) cloned, sequenced and mapped the gene encoding bovine herpes virus type 1 glycoprotein gIII (gC). Comparison of the BoHV-1 gIII amino acid sequence with the homologous glycoproteins of other alphaherpesviruses revealed significant homology in the carboxy-terminal half of the molecules, including six invariant cysteine residues.
Tikoo et al. (1990) Cloned and sequenced the gene encoding bovine herpesvirus 1 glycoprotein gIV (gD). Comparison of the BoHV-1 amino acid sequence with the homologous glycoproteins of other alphaherpesviruses, including herpes simplex virus type 1 glycoprotein gD, revealed significant homology in the amino-terminal half of the molecules, including six invariant cysteine residues.

Mittal and Field (1989) isolated five thymidine kinase (tk) deficient mutants (B1 to B5) of BoHV-1 by selection for resistance to the nucleotide analogue bromovinyldeoxyuridine. The tk genes from wild type and the tk mutants B1 to B5 were cloned and sequenced using eight unique synthetic primers from a published sequence. The BoHV-1 tk gene sequence for the strain 6660 contained some differences compared to previously published for strain LA. Alignment of the predicted amino acid sequence of the BoHV-1 tk polypeptide with different herpesvirus thymidine kinases revealed five strongly conserved regions and also identified putative functional relationships with other enzymes.

Khadr et al. (1996) identified and sequenced a gene equivalent to the gK encoding gene of other herpesviruses. The primary translation product was predicted to comprise 338 amino acids and to exhibit a molecular mass of 37.5 kDa. It possesses characteristics typical for membrane glycoproteins including a potential cleavable signal sequence, three transmembrane domains and two potential N-linked glycosylation sites. Comparison to the gK proteins of the other herpesviruses revealed aa sequence homologies of 46 %, 44 %, 53 %, 43 % and 46 % with the gK counterparts of HSV-1 and 2, equine
herpesvirus 1 (EHV-1), Marek's disease virus (MDV) and varicella zoster virus (VZV), respectively.

Schwyzer et al. (1996) reported the nucleotide sequence of a 31-kb segment at the left genome end of BoHV-1 and showed that it had comprised of 19 different open reading frames (ORFs).

Meyer et al. (1997) determined the nucleotide sequence of a 10.5 kb region (map position 0.332 to 0.410) of BoHV-1. This region contained three open reading frames homologous to herpes simplex virus DNA polymerase catalytic subunit (DNApol, UL30), major DNA-binding protein (MDBP, UL29) and ICP18.5 assembly protein (ICP18.5, UL28). They showed a high homology with alpha herpes virus homologs despite large differences in the G+C content of the UL30-UL28 segment ranging from 44.4 % for varicella zoster virus to 71.5 % for BoHV-1.

Hyun et al. (1998) cloned and sequenced the gIV gene of a Korean isolate from imported cattle quarantined in Pusan and compared it with the genetic characteristics of the reference strains. The nucleotide sequence of the gIV gene of PQ strain was 100% and 98.3% homologous to those of the Cooper (Colorado-1) and ST strains, respectively.

Fuchs et al. (1999) detected BoHV-1 in whole-blood samples derived from naturally infected cattle using PCR assays specific for glycoprotein B (gB), gC, and gE of BoHV-1. The PCR products obtained were cloned and sequenced. The amplified parts of the gB, gene was found to be 100 % identical to the published sequences of BoHV-1.1 Cooper (accession no. M21474). Compared to BoHV-1.2 P8-2 (accession no. M23257)
the sequence of the amplified gB region of strain LA differed at one nucleotide (99% identity), which also led to one different amino acid (at position 114; S to T).

Ros and Belak (1999) cloned the partial glycoprotein B (gB) and D (gD) genes from five ruminant alphaherpesviruses, bovine herpesvirus 1 (BoHV-1), bovine herpesvirus 5 (BoHV-5), caprine herpesvirus 1 (CapHV-1), cervine herpesvirus 1 and rangiferine herpesvirus 1. The nucleotide sequence alignments revealed a highly conserved gB gene, with homologies ranging between 87.2 and 99.6%. The phylogenetic analysis of the gB and gD nucleotide and deduced amino acid sequences revealed that BoHV-5 is the most closely related virus to the BoHV-1 subtype 1 and BoHV-1 subtype 2 cluster and that CapHV-1 is the most distantly related virus. They described that the genetic relatedness at nucleotide level between BoHV 1.1 and BoHV 1.2 was 98.9%.

Sivarama et al. (1999) amplified a 680 bp region of the glycoprotein gene gIV by polymerase chain reaction. The PCR product was cloned and sequenced in order to identify the origin of an Indian isolate of BoHV-1. Comparison of these sequences with the corresponding one of a European strain of BoHV-1 (Cooper) revealed more than 99% nucleotide homology.

Praveen et al. (1999) attempted the expression of BoHV-1 gC gene in mammalian cells. The 2.4 kb BamHI-EcoRI fragment, containing complete coding sequence of the gC gene was excised from a recombinant plasmid, cloned and sequenced.

Arce et al. (2002) evaluated the restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of BoHV-1 and BoHV-5. They found that
monoclonal antibodies raised against BoHV 1.1 or BoHV 1.2 cross reacted with both subtypes and have explained that MAbs cannot differentiate BoHV-1 subtypes because of their very high degrees of homology shared between them.

Amplification and cloning of the 5´ and 3´ gE flanking regions were performed by Franco et al. (2002) to obtain the gE deletion fragment from a Brazilian strain of bovine herpesvirus type 1.2a (BoHV-1.2a). The predicted sizes of 5´ and 3´ gE flanking regions were 1100 base pairs (bp) and 900bp, respectively. The data obtained was compared with expected fragments based on previously reported BoHV-1 sequences. The deletion fragment was about 1900 bp in total, corresponding to the 5´ and 3´gE flanking regions, separated by 80 nucleotides, which correspond to the plasmid polylinker. This 80 nucleotides fragment harbored two EcoRI sites, one immediately downstream the 5´gE flanking region and one immediately upstream the 3´gE region.

Mahony et al. (2002) cloned the complete genome of BoHV-1 strain V155 cloned in a bacterial artificial chromosome (BAC). This was the first infectious clone constructed for BoHV-1. It was demonstrated that when maintained as a BAC, the BoHV-1 clone is stable through multiple generations of the host strain E. coli DH10B. The stability was evaluated by restriction enzyme analysis and also through the reconstitution of infectious BoHV-1 following transfection into bovine cells.

Ros and Belak (2002) identified and sequenced the complete open reading frame and promoter region of glycoprotein B (gB) gene of ruminant herpes viruses. They located to important B cell epitopes in this region, making this as the most important
immunogenic region of herpes viruses. They described gB gene as one of the most conserved region between different herpes virus species.

Patil et al. (2006) cloned a partial gB gene of the Indian isolate of BoHV-1. The region was amplified by PCR and was cloned in pGEM-T easy vector. The sequences on comparison with available sequences was found 100% homologus with Switzerland isolate and 98.7% homologous with Brazilian isolates.

Esteves et al. (2008) amplified carboxy-terminal region of the BoHV-1 and BoHV-5 gC gene and sequenced. They described this region as a good choice for analyzing the genetic relatedness of the BoHV-1 and BoHV-5, which allows the molecular differentiation and classification of herpes viruses as BoHV-1.1, BoHV-1.2 or BoHV-5.

Gabev et al. (2009) cloned the viral genomes of BoHV-1 and BoHV-5 as infectious bacterial artificial chromosomes (BACs). Two viral genomes of BoHV-1 and BoHV-5 cloned as BACs were accessible to the tools of bacterial genetics. The ability to easily manipulate the viral genomes on a molecular level in future experiments lead to a better understanding of the difference in pathogenesis induced by these two closely related bovine herpes viruses.

Jain et al. (2009) sequenced 459 bp of gB-gene based PCR products of BoHV-1. The sequences of field isolates matched completely with the sequence of cooper strain of BoHV-1.1.
Lojkic et al. (2011) carried out the molecular and phylogenetic characterisation of BoHV-1 isolated from nasal swabs collected from naturally infected cattle. They sequenced gC specific PCR products and grouped their isolates to BoHV subtype 1.1 cluster based on phylogenetic analysis.

Ranganatha (2011) amplified 578 bp gC gene of three field isolates of BoHV-1. The amplicons were cloned in pGEMT vector, transformed in high efficiency E. coli cells and the insert was confirmed by Eco R1 digestion. The sequence and alignment homology scores for field samples indicated 100 % homology between all isolates and had more than 70 % similarity among the BoHV-1.1 reference sequences.

Surendra et al. (2011) studied the molecular characters of BoHV-1 viruses circulating in India by cloning, sequencing and Hind III restriction endonuclease enzyme analysis of twenty two BoHV-1 virus isolates collected from different regions of the country. They targeted U₅ 1.67 and U₄ 44 regions of the virus. They found that all the isolates belonged to BoHV-1.1 and were 95 % -100 % homologous with reference cooper strain of BoHV-1. None of the 22 isolates showed similarity to BoHV-1.2a, 1.2b or BoHV-5.

2.6 Real time PCR

The real time polymerase chain reaction is a recent modification to PCR that is rapidly changing the nature of biomedical science research to be conducted. It was first introduced in 1992 by Higuchi and co workers and since then, it has seen a rapid increase in its use. Real time PCR allows precise quantification of specific nucleic acids in a complex mixture even if the starting amount of material is at a very low concentration.
This is accomplished by monitoring the amplification of target sequence in real time using fluorescent technology. How quickly the amplified target reaches a threshold detection level correlates with the amount of starting material present (Higuchi et al., 1993).

Lovato et al. (2003) studied the infection of cattle with a Bovine herpes virus -1 strain containing a mutation in the latency-related (LR) gene. They standardised a TaqMan real time PCR to quantify the virus. Real-time PCR analysis indicated that lower levels of viral DNA were present in the trigeminal ganglion of calves infected with the LR mutant throughout acute infection. These results had suggested that the antiapoptotic properties of the LR gene play an important role during the establishment of latency.

Abril et al. (2004) studied the factors that contribute to neurovirulence of Bovine herpes virus -1 in interferon receptor deficient mice. They had designed primers and probes for quantitative real time PCR using Primer express software of Applied Bio (ABi) systems. The designed set of primers and probe amplified a 97 bp genome on the conserved gB gene of the virus. The amplifications were performed as per standard protocols in a 25 μL reaction mixture. The data were analysed on an ABi PRISM 7700 detector with the appropriate sequence detector software (version 1.6). The sensitivity and specificity were essentially determined using standard programmes.

Wang et al. (2007) validated a real-time polymerase chain reaction assay developed for detection of the presence of bovine herpesvirus type 1 in extended bovine semen. The assay detected a region encoding a highly conserved glycoprotein B gene and used the primers and probes designed by Abril and co workers in 2004. The real-time
PCR assay was validated for specificity, sensitivity and repeatability using spiked semen and semen from naturally infected animals. It was found that the real-time PCR was very rapid, highly repeatable and more sensitive than conventional virus isolation method for the detection of BoHV-1 in extended semen. This assay was accredited by the OIE and has been recommended to be adopted as a prescribed test for international trade. They also advocated chelex 100 resin based extraction method as the best way to isolate DNA from semen samples.

Wang et al. (2008) further elaborated their earlier works as stated above by validating the primers and protocols in an international level inter-laboratory collaboration for a ring trial on screening semen samples. They found that the real time PCR assay developed by Abril et al. (2004) and validated by these authors in 2007 to be more than 99% sensitive and more than 90% specific in detection of BoHV-1 in semen in relation to virus isolation.

Brown et al. (2008) developed a real time PCR using the primers and probe designed targeting a conserved region on the thymidine kinase gene of BoHV-1. The standardised assay was directed towards diagnosis of Bovine herpes virus-1 associated with encephalitis in aborted foetus. The assay specifically detected the virus in 12 foetuses which showed neurological disorders.

Zou et al. (2010) developed a real-time quantitative assay for rapid and sensitive quantification of glycoprotein C gene of Anatid herpes virus-1. The assay offered an attractive method for detection of the virus and the investigation of distribution pattern of the virus in vivo and molecular epidemiological studies.
Laszlo et al. (2011) during their study on pathogenesis of intra-uterine transmission of Bovine Herpes Virus-4 in bovine foetuses, they had employed Real time PCR for detection and quantification of Bovine Herpes Virus-4 in the blood, infected bovine semen and spleens of still borne calves. They found that BoHV-4 was transmitted without production of antibodies owing to the low immunogenicity of the viral proteins.

Paul and Timothy (2011) developed a multiplex Real time PCR using TaqMan probes and primers for simultaneous detection of three important viral pathogens of bovine respiratory disease complex (BRDC) viz., BoHV-1, Bovine viral diarrhoea virus and Bovine parainfluenza-3 virus. The assay was optimized and validated using cell culture infected material and bovine clinical samples collected from BRDC cases. The sensitivity of the test was analysed by comparing the multiplex with singleplex assays.

Diallo et al. (2011) developed a duplex real time PCR for the detection and differentiation of two closely related BoHV-1 and BoHV-5 targeting DNA polymerase gene of BoHV-1 and BoHV-5. The assay had detected twenty two BoHV-1 and six BoHV-5 collected from different countries across the world. The duplex assay had the detection limit of 10 copies of BoHV-1 and 45 copies of BoHV-5 in spiked semen and brain samples with reaction efficiencies of 1.04 for BoHV-1 and 1.08 for BoHV-5.

Rana et al. (2011), further validated the protocols developed by Abril et al., (2004) and Wang et al. (2007) by applying their real time PCR assay on frozen semen samples of cattle and buffaloes. They found that the primer and probe developed by Abril et al. (2004) to be 100 % sensitive and 90.04 % specific in detection of BoHV-1 in semen when compared to virus isolation. They found that, out of 574 cattle and buffaloes that
were seropositive to boHV-1 antibodies, 1.97 % semen batches from cattle and 3.36 % semen batches from buffaloes were positive for BoHV-1 by real time PCR.

In India, thus far, there are no literature available on development of Real time PCR for detection of BoHV-1 in semen or/ and any other clinical samples.

2.7 Real time PCR antigen detection kit

From 2011 onwards, based on the primers/ probe designed by Abril et al. (2004) and further validation of these primers by Wang et al. (2007), two kits have been marketed in the European market. Life Technologies Inc. United kingdom (UK) is marketing a kit under the trade name “VetMAX™ BoHV-1 Kit” for detection of BoHV-1 in semen and other clinical samples. The other kit is marketed by PrimerDesign™ Ltd under the trade name “Genesig”. Both these kits are based on the protocols described by Wang et al. (2007). Each kit costs more than one lakh Indian rupees.