Discussion
V. DISCUSSION

India presently stands first in milk production which is mainly due to extensive cross breeding programmes undertaken in the past four decades. This indiscriminate cross breeding programme with foreign germplasms and resulting in increased milk production has rendered the Indian cattle more susceptible to newer diseases. Bovine herpes virus-1 has been one of the most important viral pathogens emerged in recent times in India, causing economic losses in the form of embryonic deaths, abortions, infertility, stillbirths and/or birth of weak calves that die within a few days.

The biggest challenge in herpes viral infections is the carrier status they induce in the animals (Lovato et al., 2003), consequent to which presence of antibody in an animal may not indicate an active infection. Considering this major drawback in serum based tests, detection of virus or its antigen becomes mandatory to designate any animal as positive for Bovine herpes virus -1 (OIE, 2010). Bovine herpes virus -1 (BoHV-1) is of significance to international bovine germplasm trade. In India, the prevalence of BoHV-1 is on an ever increasing trend, with the latest seroprevalence report recorded at 42 percent during the year 2010-11 (PD_ADMAS annual reports, 2010-11). Compared to the huge prevalence of BoHV-1 and large cattle population of India, the research work carried out on the molecular epidemiology of the virus and characterization of the circulating BoHV-1 strains in the country, is very little. There is an urgent need to study the circulating strains of BoHV-1 in India to add vital epidemiological data on this disease so that effective control measures can be instituted. In view of nearly half of cattle population in the country being seropositive for BoHV-1 antibodies and poor
infrastructure facilities at state level laboratories for virus isolation/antigen detection, there is a need for the development of a more sensitive diagnostic test and its evaluation with available tests to assess the practical relevance in clinical diagnosis of BoHV-1 infections in animals. This evaluation becomes more critical since the entire developing world including India records high seropositivity in spite of absence of vaccination against BoHV-1. Further, this is of highest significance in animals in breeding bull stations and/or bull mother farms, which are the epicentres of breeding programmes and sexually transmitted diseases.

5.1 Collection of samples

Samples were collected from 212 animals from four organized farms and field outbreaks suspected of BoHV-1 infections (Table 1). The sample collection in organized farms was made exclusively in the early mornings which recorded sudden change of weather conditions like sudden rains and adverse cold weather conditions. It has been demonstrated that the corticosteroids released naturally due to stress or administered intentionally causes reactivation of latent BoHV-1 virus in carrier animals (Belknap et al., 1994; Hage et al., 1996 and Caron et al., 2002). In animals, sudden change of weather is a major stress factor which naturally induces release of corticosteroids, which may reactivate the latent virus into shedding. The selection of season for sample collection during our study was in accordance with the reports of Singh et al. (1985) who found that intense winter as the most suitable season for collection of clinical samples from BoHV-1 carrier animals for virus isolation and they have also reported that, in summer BoHV-1 is relatively inactive.
The clinical findings of conjunctivitis ranging from mild, purulent lacrimation to reddened conjunctiva to severe corneal opacity in animals with BoHV-1 (Plate 1) were in agreement with works of Mehrotra et al. (1976) who isolated the first BoHV-1 in India from a calf suffering with keratoconjunctivitis and Mohankumar et al. (1994) who isolated the first BoHV-1 in Karnataka state from a cow suffering with keratoconjunctivitis. Further, Ravi (2009) and Ranganatha (2011) have reported ocular lesions in BoHV-1 ailing animals. The respiratory symptoms of moderate cough, nasal discharges ranging from serous to mucopurulant, ulcerations in the nostrils with foul smelling expirations with typical red nose presentations (Plate.1) were in accordance with most of the researchers who have described respiratory form of BoHV-1 infections (Mehrotra et al., 1976; Singh et al., 1985, Mohankumar et al., 1994; Murphy et al., 1999; Ravi, 2009 and Ranganth, 2011). In the present study it was found that only a few animals had either respiratory or conjunctival symptoms/lesions but most of them had symptoms involving both respiratory as well as ocular forms.

Though, BoHV-1 is a major pathogen causing various forms of reproductive disorders, there has been not much work done on this aspect, hence in the present study more importance was given to reproductive disorders caused by BoHV-1. The symptoms of vulvovaginitis with varied degrees of pustular lesions on the vulval mucosa visualised during the study and the history of abortion in most of the cases that were presented with lesions of vulvovaginitis (Plate 2) were in agreement with earlier findings of Mehrotra et al. (1994) who described a storm of abortions due to BoHV-1 infections in an organised farm in Haryana, India. Dhand et al. (2002), Konrad et al. (2003), Koppad et al. (2007) and Ravi (2009) have also reported characteristic lesions of vulvovaginitis and abortion.
as the important symptoms in BoHV-1 genital tract infections in cattle. Majority of the animals from which samples were collected in organised farms did not have lesions of vulvovaginitis however; a few of them had history of abortions.

Majority of the breeding bulls from which samples were collected during this study did not have any symptoms or lesions characteristic to BoHV-1 infections. This was in confirmation with the works of Hage et al. (1996) who induced stress to a seropositive animal with dexamethasone, the animal did not produce any symptom of the disease/infection, but the swabs collected after dexamethasone treatment yielded the isolation of the virus in cell cultures, highlighting the subclinical status of BoHV-1 in animals. However, during our sample collection, a countable number of breeding bulls had lesions of congestion and ulcerations of the penile mucosa. One bull had pustular lesions on the penis (Plate 3). Our findings were in agreement with descriptions of Gee et al. (1996) and Murphy et al. (1999) who have described pustules on penile mucosa in BoHV-1 infections and Pandey et al. (2000) who recorded an outbreak of balanoposthitis in an artificial insemination centre in Uttar Pradesh, India.

The reproduction ratio of an infectious agent is defined as ‘the average number of secondary cases generated by one primary case in a wholly susceptible population of defined density’. The reproduction ratio for BoHV-1 is estimated to be at least 7, in a dairy cattle herd (Hage et al., 1996 and Benoit et al., 2007). Hage et al. (1996) who has done a classical study on population dynamics of BoHV-1 has aptly explained how BoHV-1 spreads in a susceptible herd. In one of the epidemiological study by Nuotio et al. (2007) in Finland, one seropositive breeding bull had transmitted the BoHV-1
infection to nine neighbouring breeding bulls in a span of one year. This explains the relative contagiousness of the virus in bovine population. Though, high yielders and foreign breeds are more susceptible (Ravi, 2009), going by the definition of reproduction ratio and pathoepidemiology of Bovine herpes virus-1, all cattle including exotic and indigenous breeds are equally susceptible for BoHV-1 infections (OIE, 2010). BoHV-1 can be transmitted by direct contact between animals at mucosal levels of respiratory system or at genital system (Benoit et al., 2007 and OIE, 2010). Airborne transmission by aerosol route has been demonstrated on short distances (Mars et al., 2000). Hence, the associations between animals in a herd/farm appears to be a key factor in spread of the virus. Keeping these key epidemiological factors in view, we had selected four organised farms which were located in and around Bangalore, having different managerial practices like feeding, watering and housing to evaluate the relative disease dynamics in different managerial practices under similar environmental conditions.

5.2 Serodiagnosis of BoHV-1 infections

Seroprevalence of BoHV-1 was studied by an indirect ELISA using Avidin - Biotin ELISA kits supplied by PD_ADMAS, Bangalore. In India this has been the most commonly used kit for detection of BoHV-1 antibodies in cattle (Suresh et al., 1999; Koppad et al., 2007 and Ravi, 2009) though several other versions of indirect ELISA have also been used by many authors (Suribabu et al., 1984; Kita et al., 1991 and Vanorischot et al., 1996). Jain et al. (2009) used indirect FAT and Monoclonal blocking ELISA for evaluation of serological status of BoHV-1 in breeding animals of Gujarat state. Nandi et al. (2010) opined that competitive ELISA detects BoHV-1 antibodies better than indirect ELISA in serum samples. Apart from these, several authors have
advocated serum neutralisation test as a better test for BoHV-1 antibody detection (Murphy et al., 1999; Ganguly and Mukopadhayay, 2010).

Avidin- Biotin ELISA kits were available locally, with proven records of better sensitivity, specificity and further these kits have been used for screening more than twenty thousand bovine serum samples for BoHV-1 antibodies, across the country at India’s national laboratory PD_ADMAS. For the purposes of efficiency and for better comparison and/or analysis of the results these kits were procured and used during our studies.

Overall, 34.90 % of the animals tested during the study were seropositive for BoHV-1 antibodies by ELISA (Table 3). Previously, the observed seroprevalence of BoHV-1 in Karnataka state were, 19.23 % (Gajendragad et al., 1997), 64.22 % (Suresh et al., 1999), 19.2 % (Koppad et al., 2007) and 16.69 % (Ravi, 2009).

More or less similar results were obtained in different states of India by Suresh et al. (1999), Chinchkar et al. (2002), Sarumathi et al. (2002) and Dhand et al. (2005) with a seropositivity of 38.01 %, 32.26 %, 27.4 % and 28.76 %, respectively.

On a global basis our results of 34.90% overall seroprevalence was comparable with the seroprevalence 33.97% (Kargar et al., 2001) in Iran in Asian continent; 25.9 % (Ghram and Minocha, 1990) in Tunisia of African continent; 37.8 % (Durham and Hassard, 1990) in Canada, 37 % (Guarino et al., 2000) in Uruguay of American continent; 35.9 % (Boelaert et al., 2000) in Belgium, 34.99 % (Castrucci et al., 1997) in
Italy, 40 % (Boelaert et al., 2000) in Netherlands, 38 % (Ackermann and Engels, 2006) in Poland of European continent.

The seroprevalence recorded in breeding animals in organised farms in the present study (Farm A, B, C and D) was 35.02%. This finding was in agreement with Jain et al. (2009) who reported 29.21 % serosity in breeding bulls of Gujarat state and Nandi et al. (2010) who have recorded 35 % of BoHV-1 seroprevalence in breeding animals in north India. The values obtained for breeding animals during our study were lower than the findings of Deka et al. (2005) who revealed 45.01 % prevalence of BHV-1 antibodies in cattle bulls of Punjab; Ganguly and Mukhopadhay (2010) have reported 48.15 % BoHV-1 seroprevalence in breeding animals of West Bengal state.

In spite of similarities between overall results obtained during the present study and previous studies; there are significant differences in prevalence rates between the results obtained from different studies in India and worldwide. This may be due to differences in the cattle populations, herd size and sampling procedures. Further, the management practices, the season and geographic region assessed greatly influences pattern of BoHV-1 seroprevalence.

In the present study, the highest seroprevalence of 56 % (Table 3) was recorded in Farm B which maintained breeding animals in a large, open sheds and were in let loose / let out, with common grazing and drinking facilities. This is attributable to the close social relationship amongst breeding animals at grazing pasture, drinking tanks and indiscriminate animal huddlings during chilly weathers in open yards which facilitates direct contact between animals for easy dissemination of the virus. The seroprevalence
was relatively low in Farm A (38.77 %) and Farm C (21.05 %) which followed better managemental practices with individual pens, with separate feeding and drinking facility for each animal, which avoids direct contacts between animals for longer times, avoiding viral entry and further disseminations. The least seroprevalance of 17.75 % was recorded in Farm D which had better managemental, hygienic and bio security practices with spacious modern individual animal pens, separate feeding and drinking facilities for each of the animals in pen.

Out of 35 serum samples collected from field outbreaks, 34.28 % were seropositive for BoHV-1 antibodies. During outbreaks, samples were collected from animals under different stages of the disease and randomly from small herds of two -three cows, no correlation can be drawn out of this finding alone, however the significance of serology in relation to other diagnostic techniques in clinical diagnosis of BoHV-1 has been detailed in future chapters.

The finding of overall seropositivity of 34.90 % for BoHV-1 in cattle during this study, is in agreement with the annual reports of India’s national laboratory PD_ADMAS, which recorded 42 % seropositivity in Indian cattle (PD_ADMAS annual report, 2011). Truly, 34.90 % is a huge percentage considering the fact that India does not practice BoHV-1 vaccinations. Possibly, this gives the larger evidence of alarmingly wide spread nature of BoHV-1 in Indian cattle. Due to herpes viral latency, an animal once infected develops antibodies and the animal remains infected carrier for life time. Whenever the carrier animal is subjected to stress conditions (disease/ change of weather/ change of feed / place, etc.,) the latent virus gets reactivated and gets excreted in the
respective secretions. So an animal positive for antibody means it harbours the virus in active form or in latent (or carrier /passive ) form, can excrete the virus naturally in active infection or whenever they are subjected to stress conditions in passive/latent infections. Hence the present study recommends the removal of the seropositive animals from the herd in a breeding station; however OIE (2010) advocates removal of animal only if positive by both antigen detection and antibody detection. Considering the nature of herpes virus pathogenesis (latency and reactivation) and the fact that there is no BoHV-1 vaccination practiced in India, the present study recommends to remove seropositive animals from breeding herd, because it is speculated that these seropositive animals will excrete the virus under stress conditions and will be source of infection to other susceptible cattle.

5.3 Isolation of Bovine herpes virus -1 in cell culture

Swab samples collected from 121 animals were subjected for virus isolation in MDBK cells. Samples from eight animals produced cytopathic changes which mainly comprised of rounding, vacoulations, thread like cellular elongations and typical “bunch of grapes” like cellular aggregations (Table 4 and Plate 4). Similar descriptions have been described by the researchers who have worked on isolation of BoHV-1 from nasal swabs of ailing cattle (Mehrotra et al., 1977 and 1994; Pritchard et al., 1997; Rola et al., 2005; OIE, 2010 and Ranganatha, 2011). Mehrotra et al. (1976) and Mohankumar et al. (1994) who isolated BoHV-1 from conjunctival swabs of cattle reported “bunch of grape like aggregation” as the most important cellular change induced by BoHV-1 in cell culture. Misra and Mishra (1987) isolated BoHV-1 from aborted materials/uterine mucus
samples and Cardenas et al. (2006) isolated 29 BoHV-1 isolates from the 49 aborted foetus samples, described typical herpes viral CPE as seen during our study.

No differences were noticed in induction, progression and completion of cytopathic changes between different isolates obtained during this study. This was in accordance with the findings of Saha et al. (2010) who reported similar cytopathic changes for their BoHV-1.2 isolate obtained from nasal swab of a BoHV-1 ailing cow in West Bengal state of India.

Semen samples collected from 91 bulls in three breeding bull stations were subjected for virus isolation. Semen sample from one bull yielded virus isolate (Table 4). Several workers have reported toxicity of seminal enzymes in cell culture and thereby suggesting dilution of neat semen with either serum or media before inoculations on to cell culture for virus isolation (Rudi et al., 1992; Engelenburg et al., 1995; Wang et al., 2007 and OIE, 2010). In the present study, inspite of using serum to neutralise seminal enzymes a few of the semen samples continued to produce extensive degeneration and detachment of cells within 24 hr in the first passage and a few even during second passage. It was practically very difficult to differentiate CPE and the seminal cytotoxicity which required more passages for a few semen samples to confirm and differentiate the CPE. Out of the 91 semen samples tested, only one sample developed cytopathic effect, which was typical of BoHV-1 as described earlier. The results were in concurrence with the findings of Rudi et al. (1992) who isolated BoHV-1 from semen of bulls ailing with Balanoposthitis. Cytotoxicity and herpes viral CPE have also been described by
Engelenburg et al. (1995) and Deka et al. (2005) during their virus isolation studies from semen samples in MDBK cells.

In the farm-wise analysis, samples collected from Farm A, C and D did not yield any virus isolates in cell cultures. In Farm B, samples collected from three cows and one bull yielded the virus isolates, thus emphasizing close physical contacts like, licking, nose to nose contacts either during common housing in large open animal sheds (since they were let loose type) or at common grazing/drinking yards (since they were let out type) will serve for better virus transmission amongst susceptible populations, as described by Benoit et al. (2007).

From field outbreaks, though thirty five animals were showing some of the classical symptoms of the disease (respiratory and/or conjunctival and/or reproductive form) samples from only five animals yielded the virus in cell culture system. This finding further adds on to the available information on poor sensitivity of virus isolation technique in clinical diagnosis of BoHV-1 in cattle. Previously, Rudi et al. (1992); Engelenburg et al. (1995); Kataria et al. (1997); Deka et al. (2005); Jain et al. (2009) and Ranganatha (2011) have categorically listed virus isolation as a test with poor sensitivity, owing to its multi stepped procedures during which virus may be lost at any step with requirement of sterile working conditions, costly reagents, skilled manpower and most importantly poor sensitivity in detecting low quantity of the virus in clinical samples.

During virus isolations from semen samples (which usually contains very minimal amount of the viral particles), since we dilute the semen with serum/media to avoid semen induced cytotoxicity, there are very high possibilities that we dilute so much that
we may miss out the portion of the semen which contains the virus (Deka et al., 2005 and Benoit et al., 2007). This is a practical difficulty/disadvantage which further adds to the poor sensitivity of virus isolation assay in detecting BoHV-1 in semen samples, though it is a gold standard and an approved test for international trade purposes (OIE, 2010).

5.4 Comparison of serology and virus isolation in diagnosis of BoHV-1.

During virus isolation, out of 74 seropositive animals, all did not yield the virus, rather samples from only four of these animals produced characteristic cytopathic changes (Table 10) and rest 70 seropositive animals were negative for BoHV-1 by virus isolation. In principle, virus isolation and development of CPE requires minimal number of viable virus particles in the collected clinical sample. A seropositive animal will excrete the virus either when in an active infection or when it comes under suitable stressors resulting in cortisone production and immunosuppression (Benoit et al., 2007 and OIE, 2010). Possibly, when we collected the clinical sample for our studies, all the seropositive animals might not have been under immunosuppression resulting in reactivation and excretion of the virus in case of breeding animals; or the amount of virus (number of viable particles) in the collected sample might not have been sufficient enough to produce the desired CPE or the virus might have been lost during dilutions/complex procedure of virus isolation process.

Interestingly, upon inoculation of samples collected from 138 seronegative animals, samples from five animals developed CPE characteristic to BoHV-1 (Table 10). This finding was in accordance with the research findings of Loewen and Darcel (1985),
Rudi et al. (1992), Gee et al. (1996), Hage et al. (1996), Deka et al. (2005), Benoit et al. (2007) and Laszlo et al. (2011). This is a very unique situation which states that seronegative animals need not be negative for the virus. This is probably attributed to the following reasons individually or in combinations. First, an animal can be sero-negative and can still yield the virus if the clinical samples are collected in the very early stage of infection before antibody production begins (Loewen and Darcel, 1985; Rudi et al., 1992 and Gee et al., 1996), because antibody production requires at least 10-14 days (Richard et al., 2007). Substantiating this, Hage et al. (1996) have demonstrated that seronegative animals can excrete virus of upto $10^7$ TCID$_{50}$/ml between onset of infection and seroconversion. Second, recent studies on herpes viruses have described special mechanisms of viral spread between cells such that they never get exposed to the host immune system (Antinone et al., 2006) so virus exists without actually coming in contact with immune effector cells. Third, the carrier status is usually established in neuronal cells/ganglions (sacral ganglion for genital tract infections and trigeminal ganglion for respiratory tract infection) which either express very low levels of MHC or never express MHC on their cell surface (Richard et al., 2007). Fourth, BoHV-I has got unique abilities to destroy the synthesis of MHC molecules in the infected cell (Benoit et al., 2007). Finally, a recent finding by Laszlo et al. (2011) has shown that because bovine herpes viral surface proteins are poorly immunogenic, a seronegative animal can still harbour and yield the virus upon isolation.

Therefore, an antibody negative animal can be positive for the virus as noticed during the study. Hence a seronegative animal especially in breeding stations should
always be subjected for antigen detection by a sensitive test like real time PCR as standardised and demonstrated during this study.

5.5 Virus Titration and Neutralisation

From the beginning of traditional virology, the α method of virus neutralisation test has been the gold standard technique for confirmation of any virus (Rovozzo, 1974) and so also for BoHV-1 (OIE, 2010). The neutralisation index is defined as the virus titre (in log10) in the presence of negative control serum minus the virus titre in presence of specific antiserum. Isolate to be confirmed as BoHV-1, should have a neutralisation index of greater than 1.5 (OIE, 2010 and Ranganatha, 2011).

In the present study, virus titration and neutralization was carried out in a 96 well cell culture plates (Plate 5) for all the isolates. The neutralization titre and neutralization index of all the isolates is depicted in Table 5. All the five BoHV 1.1 isolates and four BoHV 1.2 isolates were completely neutralised by BoHV-1 hyper immune serum obtained from PD_ADMAS. Further, all the nine isolates had a neutralisation index of more than 1.5 confirming them as specific BoHV-1 isolates. Similar neutralisation findings have been reported by previous workers for BoHV-1.1 (Deka et al., 2005 and Ranganatha, 2011) and for BoHV 1.2 (Saha et al., 2010). Since, the difference between BoHV 1.1 and BoHV 1.2 at molecular levels is minimal, both the subtypes will cross react with either of the hyper immune sera (Ros and Belak, 1999; Arce et al., 2002 and Saha et al., 2010).

From amongst the nine isolates, the isolate obtained from semen sample had the lowest titre of $\log 10^{2.24}$ TCID$_{50}$/0.1ml (Table.5). This could be because a very small
fraction (about 0.5 ml) of the one complete ejaculate (about 7 to 8 ml) was taken for virus isolation, which was further diluted with serum (to avoid cellular toxicity) when inoculated to in cell culture. It could also be because of low viral load in the semen itself (Deka et al., 2005 and Benoit et al., 2007). The isolate derived from conjunctival swab had the highest titre of $10^{7.24}$ TCID$_{50}$/0.1ml (Table 5) which is in confirmation with the findings of Mehrotra et al. (1976); Mohankumar et al. (1994) and Ranganatha (2011), who also have isolated BoHV-1 with relatively high titres from conjunctival swabs. The high virus titre in conjunctival swabs could be because, the lacrimal secretions stays for a longer time in the lacrimal fossa of the eye and there will be a gradual concentration of the virus at this region compared to other areas from where samples are collected (nose, vagina and penis) where the secretions tend to continuously fall off due to gravity, resulting in low titre of the virus.

5.6 Extraction of viral DNA

Extraction of viral DNA from clinical swabs and tissue culture supernatants that showed CPE was done using QIAamp DNA Mini Kit. These kits were highly efficient by the fact that it took about 40 min to complete the entire procedure of DNA extraction. The recovery of DNA in this study was 24.5 ng/µl and 53.6 ng/µl in swab samples and tissue culture supernatants respectively. This was in accordance with findings of Ranganatha (2011) who extracted 60 µg/ml of DNA from clinical swabs using similar kits. Deka et al. (2005) and Jain et al. (2009) also used similar kits for extraction of BoHV-1 DNA from clinical samples and have opined that these kits were highly efficient in extracting more DNA from clinical as well as tissue culture samples.
In this study the Chelex extraction method yielded an average concentration of 37.1 ng/µl of DNA in semen samples. It was found that, this extraction method was very simple, fast, low-cost, sensitive and reliable method for the preparation of DNA from bovine semen for PCR assays. This is in confirmation with many previous works using DNA from bovine semen (Walsh et al., 1991; De Lamballerie et al., 1992; Zandotti et al., 1993; Wiedmann et al., 1993; Grom et al., 2006; Wang et al., 2007 and OIE, 2010).

Presence of PCR inhibitory substances in bovine semen has been exhaustively explained by many previous workers (St-Laurent et al., 1994; Wiedmann et al., 1993; Wang et al., 2007 and Manojkumar et al., 2011). Thus selection of an efficient DNA extraction method was very critical for the successful PCR detection with better sensitivity. It has been found that chelating resin-based extraction produces the best results in terms of sensitivity and consistency. World organisation for animal health (OIE, 2010) has approved this resin based DNA extraction method for DNA isolation from semen samples. Chelex preparation of the samples results in less inhibitory effects, leading to more successful PCR amplification than with traditional methods, due to the protection of DNA by Chelex 100 at high temperature and by inactivation of polymerase inhibitors during described DNA extraction protocol (Walsh et al., 1991; De Lamballerie et al., 1992 and Zandotti et al., 1993). Considering these findings, the Chelex resin based method was adopted for isolation of DNA from semen samples during the study.

5.7 Confirmation of BoHV-1 isolates by gB gene based conventional PCR

The nine isolates obtained during this study were confirmed by molecular methods, wherein, DNA extracted from these nine isolates was subjected to conventional
PCR and for further phylogeny. For this, primers targeted against the most conserved region of the virus were used.

Upon conventional PCR, the extracted DNA from all the nine MDBK cell culture supernatants that showed herpes virus specific CPE, yielded the desired amplicons of 443 bp further confirming the isolates as BoHV-1 (Fig. 3).

Glycoprotein B is an essential component of BoHV-1, which plays a major role in virus entry. It also represents a dominant antigen that induces protective immunity in the natural host. Glycoprotein B gene has been the most commonly used gene to study the genetic relationship among herpes viruses, as sequence data are available from a large number of herpes viruses. Therefore, the comparison of gB gene sequences can be readily used to estimate the genetic relationship of newly characterised herpes viruses. The complete genome sequence analysis of gB gene of herpes viral isolates from several ruminant species has shown gB gene as the most remarkably conserved region among herpes viruses (Ros and Belak, 2002). Many authors have compared different genome targets like gB, gC and gD regions for conventional PCR and have found that gB gene targeted PCR to be the most efficient target for clinical diagnosis of BoHV-1 in animals (Vilcek et al., 1995; Kataria et al., 1997; Ros and Belak, 1999; Jain et al., 2009 and OIE, 2010). Further, several authors from across the world have shown that primers designed by Ros and Belak (1999) amplifying a region of 443 bp on the gB gene of BoHV-1, is specific to the target and is present on all BoHV-1 isolates (Patil et al., 2006; Jain et al., 2009; OIE, 2010 and Ranganatha, 2011). Considering these facts, the set of primers designed by Ros and Belak (1999) amplifying a region of 443 bp on the gB gene
of BoHV-1 were selected for molecular confirmation of the isolates by conventional PCR and subsequent nucleotide analysis for phylogeny.

5.8 Cloning, sequencing of the PCR products and phylogenetic analysis.

The gB specific PCR products from nine isolates obtained during this study were cloned in pGEM T vector and were sequenced from Eurofins Biotech Pvt Ltd, Bangalore, India. The nucleotide sequences obtained were compared with the published nucleotide sequences of gB gene of BoHV-1 from NCBI nucleotide sequence data base. Alignment studies were performed using the sequence analysis software program MegAlign of DNA Star. In the present study the orientation of the cloned product in the vector was confirmed by EcoRI digestion with the release of 443 bp products from 1.7 kbp pGEM T vector (Fig. 4). Similar strategy for cloning of the partial genome of BoHV-1 has been demonstrated by Gupta et al. (1999); Patil et al. (2006) and Ranganatha et al. (2011), wherein the orientation of the insert in the plasmid vectors was confirmed by restriction analysis with EcoRI digestion. Sequence analysis was performed with the Lasergene 6 package (DNASTar Inc., Madison, USA). Phylogenetic analyses of the 443-bp fragment BoHV-1 gB were conducted using MEGA version 5 (Tamura et al., 2007) using the maximum parsimony method with 500 bootstrap replicates. The tree was constructed with the modules of MEGA 5 programme.

Sequence data of gB gene of the nine isolates as presented in (Fig. 4 and 5) clearly indicated that they are indeed BoHV-1. Five of these nine isolates KVAFSU BNG-1, KVAFSU BNG-2, KVAFSU BNG-3, KVAFSU BNG-4 and KVAFSU BNG-9 were clustered under BoHV-1.1 group. Further, the other four BoHV-1 isolates,
KVAFSU BNG-5, KVAFSU BNG-6, KVAFSU BNG-7 and KVAFSU BNG-8 were grouped under BoHV-1.2 cluster.

The isolates KVAFSU BNG-1, KVAFSU BNG-3, KVAFSU BNG-4 and KVAFSU BNG-9 were more than 99.1 % identical with reference PD_ADMAS strain. Divergence of these isolates with reference PD_ADMAS strain ranged from 0.7 to 0.9 %. All these isolates had homology of more than 99.8 % within themselves with very minor differences. Inspite of having nucleotide sequences similar to other BoHV 1.1 isolates obtained during this study, KVAFSU BNG -2 isolated from bovine semen had 98 % sequence identity and 0.9 % divergence with reference PD_ADMAS strain.

The finding of presence of BoHV 1.1 in clinical samples was in accordance with the works of Patil et al. (2006) who have reported BoHV1.1 as the most prevalent subtype in India and found that PD_ADMAS BoHV-1 isolate had 100 % homology with Switzerland isolate and 98.7 % homologous with Brazilian isolates. Ranganatha (2011) isolated three BoHV-1 isolates from clinical swabs of animals ailing with respiratory symptoms and characterised them as BoHV-1.1. Jain et al. (2009) characterised BoHV-1 isolates obtained during their study in Gujarat state of India, as BoHV-1.1. Surendra et al. (2011) in a detailed work, 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 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.
During the entire study period ‘PD_ADMAS BoHV-1 isolate’ was used as a positive control. This was mainly because ‘PD_ADMAS BoHV-1 isolate’ was isolated from Hassan district of Karnataka state during 1994. The phylogenetic analysis of genome sequences in relation to this isolate provides better information on molecular epidemiology of Bovine herpes virus -1 in Karnataka state. Further, this has been one of the most commonly used viruses for comparative genome sequence analysis of BoHV-1 across India (Jain et al., 2009 and Ranganatha, 2011). Cloning and sequencing of the partial gB gene of ‘PD_ADMAS BoHV-1’ isolate by Patil et al. (2006) is the only gB gene based nucleotide sequences of an Indian BoHV-1 isolate available at NCBI for comparison purposes.

The four BoHV-1 isolates KVAFSU BNG-5, KVAFSU BNG-6, KVAFSU BNG-7 and KVAFSU BNG-8 recovered from samples collected from four different female cattle showing typical symptoms of pustular vulvovaginitis under filed conditions in an outbreak in a single village were 100% homologous between themselves and shared a sequence identity of 100% with an European strain of BoHV 1.2 (GenBank accession number AF 078725.1). However, these four isolates shared a sequence identity of 98.9% and divergence of 1.1 with PD_ADMAS BoHV-1.1 isolate, without any changes in induction of CPE in cell cultures, showing that, there is possibly a very minute difference between 1.1 and 1.2 types of BoHV-1 in Indian cattle. This finding is in correlation with works of Ros and Belak (1999) who have sequenced the gB gene and have recorded 98.8% homology between BoHV-1.1 and BoHV 1.2. Further, this is in accordance with works of Arce et al. (2002) who have shown that monoclonal antibodies (MAbs) against either BoHV-1.1 or BoHV 1.2 will cross react with each other and MAbs cannot
differentiate the subtypes indicating high degrees of similarity between BoHV 1.1 and BoHV 1.2. This is the first molecular study on BoHV 1.2 in India, although there are reports available on the isolation of BoHV-1.2 from clinical samples in India. Previously, Saha et al. (2010) have reported isolation of BoHV-1.2 in cattle ailing with respiratory tract infections in west Bengal state of India. As on date no sequences of Indian isolates of BoHV 1.2 are available on NCBI website. Hence, present study further augments circulation of BoHV-1.2 in India and could be one of the main reasons for increased herpes viral abortions in cattle in recent times.

Today, approximately 25 % of the Indian agricultural gross domestic product is contributed by the livestock sector and this tremendous growth in dairy sector is primarily attributed to the extensive cross breeding programme undertaken during the periods of “Indian white revolution in 1970’s and 1980’s” using exotic breeds or their germplasms, especially from European breeds of cattle. As mentioned in earlier chapters (chapter 2.3.4) prevalence of BoHV-1 has been well documented in European counties during the last century. Probably, the high genetic relatedness of the virus isolates obtained during this study with European strains of BoHV-1 could well support the possibility that BoHV-1 must have entered during that phase of extensive dairy development in India using foreign germplasm. Similar transmissions through germplasm/ breeding bulls have been reported by Nuotio et al. (2007) who have observed that one of the seropositive bulls imported from Denmark in 1968 introduced the BoHV-1 to Finland, contributing to further spread of the infection to a notifiable level by 1990.

However, as only a small number of BoHV -1 and its subtypes have been characterised in India, sampling errors must be considered, since the isolates examined in
this study may not be actual representatives for the more prevalent viruses found in the country. On the other hand the PD_ ADMAS strain and European strain of BoHV 1.2 which are considered as reference strains may not be the actual representatives of most BoHV 1.1 and BoHV 1.2 subtypes circulating in the country, thus it is likely that variations between strains may be larger than what is shown in this study.

The similarities of more than 99 % between the isolates obtained during this study and with the reference strains further strengthens the fact that the genome selected during this study on the gB region of BoHV-1 is remarkably conserved in all subtypes of BoHV-1 which is in accordance with numerous previous works as discussed earlier and with special reference to the works of Ros and Belak, (1999 and 2002). This finding justifies the selection of gB region for designing of primers and probe for standardisation of real-time PCR based antigen detection assay and development of the kit during this study.

5.9 Real time PCR

A real-time PCR assay was developed and standardized for the detection of BoHV-1 from suspected clinical samples including bovine semen. Validation of the developed PCR assay was performed.

5.9.1 Selection of TaqMan Probe based Real time assay

As stated previously, conventional PCR assays have been applied for the detection of BoHV-1 in bovine semen and other clinical samples. This method when compared to virus isolation is inexpensive, faster and more sensitive in detection of BoHV-1 in clinical samples (Engelenburg et al., 1993; Wiedmann et al., 1993; Vilcek et
The major drawbacks of conventional PCR is that it is prone for contamination especially during postamplification handling of PCR products, therefore utmost precautions have to be taken to prevent false positives (Wang et al., 2007; OIE, 2010; Rana et al., 2011 and Cordoso et al., 2012). This major flaw of cross-contamination in conventional PCR has been significantly reduced by a new variant of PCR technique called the real-time PCR, which is rapid, highly repeatable, purpose-specific and more sensitive than virus isolation in detection of BoHV-1. Real time PCR assay differs from the conventional PCR in that they include fluorescent reporter molecules that increase proportionally with the increase of DNA amplification in the thermalcycler which facilitates the amplification and detection of target, conducted simultaneously avoiding postamplification PCR product handling, which significantly reduces the risk of contamination. There are two types of fluorescent chemistries for this purpose. Double strand DNA binding dyes and fluorescent labelled sequence specific probe or primer. SYBR Green dye is commonly employed DNA binding dye in real time PCR assays. The disadvantage of SYBR Green dye is that it binds non specifically to any DNA in the reaction mixture (including primer-dimmer), hence requires extensive optimizations and melting point curve determination. Owing to these disadvantages, though it’s cheaper, SYBR Green real time assays are not recommended when applied for amplification of rare transcripts or low level pathogen detection in highly valued animals, like detection of BoHV-1 in pedigreed, highly valued breeding bulls and bull mothers.
On the other hand, the probe based real time PCR assays involves a single, fluorophore labelled, sequence-specific oglionucleotide, which otherwise acts like a third primer. These attributes gives the probe based real time PCR the better sensitivity, leading to specific detection of the target on a real time basis without cross contaminations. Due to these advantages, though it’s relatively costly, probe based real time assays are highly recommended for amplification of rare transcripts or low level pathogen detection in highly valued animals, like detection of BoHV-1 in pedigreed, highly valued breeding bulls and bull mothers.

5.9.2 Designing of primers and probe

In the present study ten sets of primers and probes targeting gB gene were designed (Table 2). A set of primer and probe amplifying a conserved region of 71 bp was selected for standardisation of real time PCR. The probe used in this study was labelled with the reporter/donor, 5-carboxyfluorescein (FAM) at the 5’ end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. The real-time PCR assay had detected viral DNA of both BoHV-1.1 and 1.2 strains, selectively amplifying a 71 bp sequence of the glycoprotein B (gB) gene. As on date, this is the shortest amplicon described for the detection of BoHV-1 in clinical samples. Abril et al. (2004) reported the first set of primers and probe with an amplicon size of 97 bp for detection of BoHV-1 and these set of primers were validated by Wang and co workers in an international inter-laboratory evaluation (Wang et al., 2008).
5.9.3 Standardisation of reaction mixture and the protocols

Optimization of different components of real time PCR during this study was done through extensive titration by checkerboard method. Because the entire process of Real time PCR assay was aimed at development of a diagnostic kit, standardisation with lower reagent consumption was one of the important criteria, as this would directly lead to a significant reduction in cost of the kit, consequently resulting in availability of BoHV-1 diagnosis at a lower cost.

Standardisation of reaction mixture was started from three pmoles to 20 pmoles of primers, per reaction; similarly the concentration of probe was titrated from one pmoles to 10 pmoles per reaction. Finally, the concentrations of 10 pmoles of primers and five pmoles of probe per reaction gave the best results which were defined as the maximum fluorescence values combined with the earliest Ct values. As on date these are the lowest concentrations of primers and probe described in a reaction mixture for diagnosis of BoHV-1 using real time PCR. This lower reagent consumption is probably attributed to shorter amplicon size, specificity of the primer and probes. Wang et al. (2007) have described the primer concentrations of 4.5 µmoles and probe concentrations of three µl per reaction in a reaction mixture. The concentration of DNA from the clinical sample to be used was titrated from one µl to 20 µl per reaction, the concentration of five µl gave consistent results. Hence this was fixed in the reaction mixture which is in accordance with the findings of Abril et al. (2004) and Wang et al. (2007) who also incorporated five µl as the concentration of template DNA from the suspected clinical samples during their real time PCR assays for diagnosis of BoHV-1. Using the set of primers and probe designed during this study, the cut off cycle at which all positives were detected, without
any non specific amplification (negative controls and No template controls) was at 40th cycle (Fig. 9). This cut off cycle (40th cycle) was in accordance with the most widely followed works of Higuchi et al. (1992) and Fraga et al. (2008) who have set the optimum cut-off cycle for all probe based Real time PCR at ≤ 40th cycle. Wang et al. (2007) who have previously standardised a real time assay for detection of BoHV-1 in semen have adopted the cut off cycle at 45th cycle. Hence, our work, though not significantly, but in relative terms reduces the total time taken and reagents consumption for the final diagnosis of a sample submitted for BoHV-1.

The real-time PCR assay had successfully detected BoHV-1 strains/isolates which include genotypes 1.1 and 1.2 isolated during this study (Fig. 12). Though we could not test other related herpes viruses, due to non availability of viruses or their DNA in India, the PCR assay standardised during this study appears to cross-react with several herpes viruses that are closely related to BoHV-1. This is not surprising, as the primers and probe of the PCR assay were selected from an highly conserved herpes virus gene, the glycoprotein B gene. Therefore, the sequences are also highly homologous to those of other genetically closely related ruminant herpes viruses, including cervine, caprine and rangiferine herpes viruses. However, these viruses would not be expected to be present in bovine semen and suspected clinical samples from BoHV-1 outbreaks, hence it is highly unlikely that we detect them from our target samples. Similar findings have been reported by Ros and Belak (1999 and 2002). Thus, the specificity of the PCR described is adequate for the desired purpose, i.e., detection of BoHV-1 bovine semen and from animals clinically ailing with BoHV-1 like infections. Additionally, as the sequences of primers and probe used for the real-time PCR are homologous to that of BoHV-5 virus,
this PCR assay would detect BoHV-5 virus as well which we could not test due to non-availability of BoHV-5 DNA in any laboratory in the country. Similar descriptions also have been made by researchers who have standardised real time PCR assay targeting the highly conserved genes of BoHV-1 in particular (Wang et al., 2007; Diallo et al., 2011 and Paul and Timothy 2011) and other herpes viruses in general (Zou et al., 2010 and Lazlo et al., 2011).

It is well established that smaller amplicons (shorter distances between forward primer, the probe and the reverse primer) adds to better sensitivity of TaqMan real time PCR assay (Wang et al., 2007; Fraga et al., 2008 and Diallo et al., 2011). Considering this, out of the ten sets of primers and probe designed during this study (Table 2), a set of primers and probe was selected targeting a short region of 71 bp (shortest amongst the ten sets designed in this study) on the conserved region of the viral genome and also because this set of primers and probes relatively had the least penalty score.

5.9.4 Sensitivity and specificity of Real time PCR assay

The real time PCR assay developed during this study was 100% sensitive in detection of BoHV-1 in clinical samples on comparison with virus isolation (Table 8). This was in agreement with most of the workers who have compared virus isolation with conventional PCR in general (Engelenburg, 1995 and Ranganatha, 2011) and real time PCR in particular (Wang et al., 2007, Wang et al., 2008 and OIE, 2010).

The sensitivity in terms of lowest detection limit was evaluated by sequential 10-fold dilutions of virus culture of one of the BoHV-1 isolates. The detection limit was determined to be 0.001 TCID$_{50}$ of the virus (Fig.10). As on date this is the best detection
limit described for BoHV-1 using any diagnostic assay. The real time PCR assay validated by Wang et al. (2007) can detect up to 0.36 TCID$_{50}$. Kataria et al. (1997) and Ranganatha (2011) have demonstrated that conventional PCR can detect virus load only up to 0.01 TCID$_{50}$. This ultra low detection limit achieved during the study is attributed to the primers and probe targeted to amplify a very short, conserved region of BoHV-1. The sensitivity of the real time PCR assay in terms of detection limit in dilution of the original sample was also evaluated using serial dilution of positive semen sample with cell culture medium (DMEM). The PCR assay was able to detect dilutions of positive semen sample up to a dilution of 1:80 (Fig. 11). Virus isolation was also performed on same dilution of the semen samples, the virus was isolated only in the dilution of 1:10 and rest of the dilutions did not yield the virus in cell culture system, indicating the genuine fact that the assay developed had a much greater sensitivity than virus isolation in detection of BoHV-1 in clinical samples. The real time assay validated by Wang et al. (2007) had detected virus dilutions of up to 1:160. In the present study the positive semen sample might had lower virus load than the one used by Wang and co-workers in 2007, leading to failure of virus detection in dilution after 1:80.

The real time PCR assay was also employed for BoHV-1 spiked semen samples, which consistently detected all positive semen samples and there was no detection of any negative semen samples tested. Further, there was no non-specific amplifications even when the assay was run upto 45$^{th}$ cycle (Fig. 13). Further, the NCBI-BLAST analysis of designed primers and probe suggested 100 % specificity of these to BoHV-1 including 1.1, 1.2 and 1.3 (now BoHV-5) strains which was further supported by specific detection of both BoHV-1.1 and BoHV1.2 strains isolated during this study (Fig. 12). On statistical
analysis, the developed real time PCR assay had 87.19 % specificity in relation to virus isolation in detection of BoHV-1 in clinical samples (Table 8). The finding of specificity of the real time PCR assay was in accordance with previous workers who have reported that real time PCR is more than 85 % specific in detection of BoHV-1 in clinical samples when compared to virus isolation (Abril et al., 2004; Wang et al., 2007; Diallo et al., 2011; Paul and Timothy 2011 and Rana et al., 2011).

5.9.5 Comparison of Real time PCR and Virus isolation

Comparative analysis of real time PCR and virus isolation in detection of BoHV-1 is one of the primary objectives of this study.

Samples collected from Farm A and C did not yield any virus isolate, upon subjecting them for virus isolation in MDBK cells and samples from four animals belonging to Farm B yielded the virus in cell culture system. On the other hand, subjecting the same clinical samples for real time PCR assay had resulted in detection of BoHV-1 in 3 out of 49, 11 out of 50 and 3 out of 38 samples from Farm A, B and C respectively. Further, of the samples collected from thirty five animals showing classical symptoms of the disease (respiratory and/or conjunctival and/or reproductive form) at field outbreaks, only five animals yielded the virus in cell culture system, whereas the real time PCR had detected BoHV-1 in samples collected from eighteen of these animals. The positives detected by Real Time PCR included all the samples that yielded virus in cell culture indicator system. This finding not only proves the poor sensitivity of virus isolation technique in clinical diagnosis of BoHV-1 in cattle (Engelenburg et al., 1995; Rudi et al., 1992; Kataria et al., 1997; Deka et al., 2005; Jain et al., 2009 and Rangantha,
2011) but also overwhelmingly supports the previous findings that real time PCR as a better test in detection of BoHV-1 in clinical samples, including semen samples (Abril et al., 2004; Wang et al., 2007; Diallo et al., 2011; Paul and Timothy 2011 and Rana et al., 2011).

The high sensitivity with an ability to detect ultra low levels of virus of up to 0.001 TCID₅₀/0.1ml and better specificity of the real time PCR assay in relation to virus isolation, clearly demonstrates real time PCR assay as the best test in detection of BoHV-1 in latent or carrier animals.

5.9.6 Comparison of ELISA, Virus isolation and Real time PCR for diagnosis of BoHV-1

Comparison of ELISA, virus isolation, real time PCR and their clinical relevance was the most important objective of the present study. Real Time PCR had detected all the samples that yielded virus in cell culture indicator system. Apart from detecting, twenty six additional samples as positive for BoHV-1. Hence the study concludes that the real time PCR is a better test for antigen or virus detection than virus isolation method. This finding supports the recommendation of OIE (2010) in prescribing real time PCR as test for antigen detection for international trade purposes.

During the present study all the seropositive animals did not yield the virus, rather only four out of nine virus isolates were from seropositive animals. Even in real time PCR which had detected 35 samples as positives, only 22 were from seropositives (Table 10). Though many of the seropositive animals did not yield the virus or its antigen but they conclusively indicated that these animals have been infected with BoHV-1 sometime
in its life time and they harbour the virus in latent or carrier status and can excrete the virus whenever they are subjected to stress conditions. Hence, the present study recommends culling of all seropositive animals especially in breeding bull stations and bull mother farms.

During virus isolation, five samples collected from seronegative animals yielded virus in MDBK cell line and further real time PCR detected BHV-1 in thirteen seronegative animals (Table 1). This finding is attributed to different molecular mechanisms describing the pathogenesis of BoHV-1 (Loewen and Darcel, 1985; Rudi et al., 1992; Gee et al. 1996; Hage et al., 1996; Deka et al., 2005; Benoit et al., 2007 and Laszlo et al., 2011). Therefore, present study reiterates that, an antibody negative animal can be positive for the virus, hence a seronegative animal (especially in breeding stations) should always be subjected for antigen detection by the most sensitive test like real time PCR as standardised and demonstrated during this study.

Benoit et al. (2007) in their elaborate review on molecular mechanisms of latency and reactivation of BoHV-1, have stated that during natural infection, a well-regulated transcription cascade of alpha, beta and gamma BoHV-1 genes leads to the activation of caspases and p53 resulting in programmed cell death and efficient virion release in permissive cells. The BICP0 IE gene plays a key role in this lytic phase. On the contrary, in latently infected cells, only the BoHV-1 region containing the latency-related transcript (LRT) (Fig. 1) is expressed leading to the inhibition of the lytic virus cycle. Thus indicating differences in lytic abilities of virus excreted during natural infection with that of the virus excreted during reactivation of latent virus. Since virus isolation is a multi
stepped protocol in which virus may be lost in any one step and also virus isolation requires minimum quantum of viable, lytic virus to yield detectable cytopathic changes, which is a major drawback of virus isolation method, contributing for its poor sensitivity. On the other hand, real time PCR which is a single step assay with an ability to detect either viable/lytic or non-viable/nonlytic virus of ultra low levels of up to 0.001 TCID$_{50}$, is arguably the best method available for detection BoHV-1 in carrier/latent animals or in animals showing clinical symptoms of the disease (Table 9 and 10).

5.10 Development of Real time PCR based antigen detection kits

The real time PCR assay standardised during this study had high sensitivity as well as specificity with the detection limit of up to 0.001 TCID$_{50}$ of the virus. Based on these results an user-friendly, real time PCR based antigen detection kit was developed. The kit was validated at reputed national laboratories following standard protocols of validation. Even at validating laboratories, the sensitivity of the kit was $\geq 99$ per cent (Plate 11).

The kit developed during this study had better detection limits, faster and required lesser reagents compared to the kits developed based on the primers designed by Abril et al. (2004) and validated by Wang et al. (2008). The two real time PCR based antigen detection kits available in the European markets are highly expensive costing more than Rupees one lakh (the higher cost is mainly towards charges for positive controls and transportation charges) and the kit developed in this study uses indigenously isolated BoHV-1 as positive controls and works out to be less than twenty-five thousands, hence
the present research work has a great field applicability for better health management of animals especially the breeding animals.

The set of primers, the probe and the reaction mixture developed during this study is only the second such descriptions (after Abril et al., 2004), in the world, which are being incorporated in the development of an antigen detection kit for diagnosis of BoHV-1 and first of its kind in Asian continent which paradoxically records the highest incidence/prevalence of BoHV-1.

**Conclusion**

World organisation for animal health (OIE, 2010) states that isolation of BoHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness but must be accompanied by seroconversion from negative to positive, because, it may, for instance, be a latent virus that has been reactivated due to stressful conditions. However, this recommendation does not appear to hold good in breeding stations, as the animal in any case will be source of infection to other susceptible animals. Considering these facts, present study draws following final conclusions. First, in breeding stations an animal positive either in antibody detection or by antigen detection should mandatorily be removed from the herd because a seronegative animal can still be positive for virus and a seropositive animal need not yield the virus all the time, as evidenced during this study. Second, selection of new breeding animals should be done by both antigen detection as well as antibody detection tests and only those which are negative with both the tests should be selected for breeding purposes. Third, every breeding animal should
be subjected for BoHV-1 screening both by antigen as well as antibody detection assays annually, preferably in winter seasons.

It’s true but a hard reality that the real-time PCR assay cannot be applied at all laboratories in developing countries like India, due to high cost of the real time PCR equipment as well as the TaqMan probe. However, under Indian (or similar to Indian) conditions, one recognised laboratory in each state/zone can have the real time facility (equipment and the assay/kit) and samples from across the state can be submitted to this laboratory for routine or periodical testing (especially the semen samples) for BoHV-1 detection. Considering this factor, the development of an antigen detection kit has the real utility for efficient diagnosis of BoHV-1 in clinical samples, especially in periodical screening of semen samples because, if unchecked, an infected bull can be a source of infection to thousands of female animals.