SEROPREVALENCE, ISOLATION AND CHARACTERIZATION OF INFECTIOUS BOVINE RHINOTRACHEITIS (IBR) VIRUS IN CATTLE OF ODISHA.

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CERTIFICATE–I

This is to certify that the thesis entitled ***"SEROPREVALENCE, ISOLATION AND CHARACTERIZATION OF INFECTIOUS BOVINE RHINOTRACHEITIS (IBR) VIRUS IN CATTLE OF ODISHA"*** *submitted in partial fulfillment of the requirements for the degree of* ***MASTER OF VETERINARY SCIENCE*** *in* ***BACTERIOLOGY AND VIROLOGY*** *of the Orissa University of Agriculture and Technology, Bhubaneswar is a faithful record of bonafide research work carried out by Dr. DIGBALAYA MOHANTY under my guidance and supervision.*

This is further certified that no part of the thesis has been submitted for any other degree or diplomа.

This assistance & help received from different sources during the course of study have been fully acknowledged.

31.7.10

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CERTIFICATE – II

This is to certify that the thesis entitled ***"SEROPREVALENCE, ISOLATION AND CHARACTERIZATION OF INFECTIOUS BOVINE RHINOTRACHEITIS (IBR) VIRUS IN CATTLE OF ODISHA"*** *submitted by Dr. DIGBALAYA MOHANTY to the* ***Orissa University of Agriculture and Technology, Bhubaneswar*** *in partial fulfillment of the requirements for the degree of* ***Master of Veterinary Science*** *in the subject of* ***Bacteriology and Virology*** *has been approved by the student's advisor committee after an oral examination on the same in collaboration with an external examiner.*

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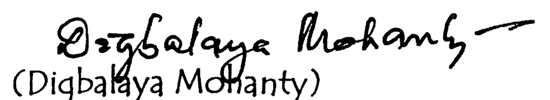

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ABBREVIATIONS

%	:	Per Cent
≥	:	Greater Than Equal
°C	:	Degree Celsius
°F	:	Degree Fahrenheit
µg	:	Microgram
µl	:	Microliter
ADMAS	:	Animal disease monitoring and surveillance unit
AP	:	Alkaline Phosphatase
Cmm	:	Cubic Millimeter
CO_2	:	Carbon Dioxide
CPE	:	Cytopathic Effect
CPV	:	Canine Parvovirus
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylene Diamino Tetra Acetic Acid
ELISA	:	Enzyme Linked Immune Sorbent Assay
EM	:	Electron Microscope
EMEM	:	Eagle's Minimum Essential Medium
et al.	:	et alii
FAT	:	Fluorescent Antibody Test
Fig.	:	Figure
FITC	:	fluoro-isothiocyanate
GM	:	Growth medium
HA	:	Haemagglutination
Hcl	:	hydrochloric acid
HI	:	Haemagglutination inhibition
hrs	:	Hours
IBR	:	infectious bovine rhinotracheitis
IPV	:	infectious pustular vulvovaginitis

IBP	:	infectious balanoposthitis
A-B ELISA	:	avidin biotin enzyme linked immunosorbent assay
TVCC	:	teaching veterinary clinic complex
CADRAD	:	centre for animal disease research and diagnosis
I/M	:	Intramuscular
I/P	:	Intraperitonial
IgG	:	immunoglobulin G
IUDR	:	5-Iodo-2-Deoxyuridine
LA	:	latex agglutination test
H&E	:	hematoxylin and eosin
IFT	:	immunofluorescent test
LAH	:	Lact Albumin Hydrosylate
MDBK	:	Madin-Darby Bovine Kidney
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
min	:	Minute
ml	:	Milliliter
MM	:	Maintenance Medium
$NaHCO_3$	:	Sodium Bicarbonate
AGPT	:	agar gel precipitation test
TAE	:	tris acetate EDTA buffer
FCS	:	fetal calf serum
bp	:	base pair
mM	:	millimolar
gC	:	glycoprotein C
dNTP	:	deoxy nucleotide phosphate
nm	:	Nanometer
OD	:	Optical Density
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction

PM	:	Post Mortem
RBCs	:	Red Blood Cells
REA	:	restriction endonuclease analysis
RFLP	:	Restriction Fragment Length Polymorphism
RPM	:	Revolution Per Minute
S/C	:	Subcutaneous
SNT	:	Serum Neutralization Test
SPF	:	Specific Pathogen Free
$TCID_{50}$	:	Fifty Percent Tissue Culture Infective Dose
TLC	:	Total Leukocyte Count
TV	:	Trypsin Versene
UK	:	United Kingdom
UP	:	Uttar Pradesh
USA	:	United States of America
v/v	:	Volume By Volume
w/v	:	Weight By Volume

Chapter-1

Introduction

1. INTRODUCTION

The Livestock sector not only provides essential proteins and nutritious human diet through milk, eggs, meat, etc. but also plays an important role in utilization of non-edible agricultural by-products. Livestock provides raw material by-products such as manures, hides and skins, blood, bone, fat, etc. Eighty percentage of rural households keep livestock for their subsidiary income, important for employment, livelihood, draught power ,nutrition, constant flow of quick and fast income and which reduces the vulnerability of agricultural production. According to estimates of the Central Statistical Organization (CSO), the value of output from livestock and fisheries sectors together, at current prices, was about Rs. 2,50,761 crore during 2006-07 (Rs.2,10,629 crore for livestock sector and Rs. 40,132 crore for fisheries). The contribution of these sectors to the total GDP during 2006-07 was 5.26%.

The livestock health is the back bone of dairy industries which has made a tremendous progress during the past five decades, consequent to the introduction of exotic germplasm to improve and upgrade the indigenous cattle population by cross breeding programme. As a result of this India now ranks in a better position in producing milk and milk products.

In spite of national economy significantly contributed by animal husbandry, dairying and improved breeding practice, the cattle population are always in stake due to health hazards caused by various bacterial and viral infections. leading to serious mortality rate ,infertility, loss in milk and meat production. Among these disease infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpes virus -1 (BHV-1) is an economically important emerging viral disease of domestic and wild cattle throughout the world including India . It is primarily associated with three clinical syndromes i.e. Infectious Bovine Rhinotracheitis (IBR), Infctious Postular Vulvovaginitis (IPV) and Infectious Postular Balanoposthitis (IPV). Besides the virus causes a wide variety of clinical syndromes, such as conjunctivitis, abortion, meningo-encephalitis, infertility, arthritis and mastitis.(Gibbs and Rweyemamu *et al.* 1977). It has relatively short

certain other bacterial infections may cause mixed infection of different systems..Incubation period is 6-14 days .

Considering the range, seriousness and rate of spreading of the infection and social aspect, this IBR is listed in section B of the directory released by *Office International des Epizootics* (OIE) (Van Oirschat *et al.*, (1993). Therefore IBR//IPV is officially recognised as having international trade significance and socioeconomic importance.

The remarkable feature of BHV-1 is its ability to become latent following primary infection. After infection of the animal by the BHV-1.2 subtype, it enters via nasal route. It replicates to high titres in mucous membranes of the upper respiratory tract, in the tonsils, and conjunctiva. It subsequently disseminates to the trigeminal ganglion by neuronal axonal transport. After genital infection, BHV-1 replicates in mucous membranes of the vagina or prepuce and becomes latent in sacral ganglia. The viral DNA remains in the neurons of the ganglia, for the entire life of the host. Stress condition such as transport, parturition, other infection, metabolic diseases and corticosteroid treatment can induce reactivation of the latent infection. Mehrotra *et al.* (1987). Consequently the virus is excreted and shaded in the environment with or without development of clinical symptoms. This becomes continuous source of infection for other susceptible animal population.

In primary infection, both humoral and cell mediated immunity to IBR infection are observed in cattle and both are important in resistance to further infection after recovery. Cell mediated immunity (CMI) is also important for recovery from infection and maintenance of resistance to further exposures of the virus. An infection normally elicits an antibody response and a cell-mediated immune response within 7–10 days. The immune response is presumed to persist for life, although it may fall below the detection limit of some tests. . Humoral immunity is the most prominent immune response, and measurement of neutralising antibodies is usually taken as infection of the animal (Ludwig and Gregersen, 1986).

Maternal antibodies are transferred via colostrum to the young calf, which is consequently protect against BHV-1-induced disease. . Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals up to 9 months old, and rarely in animals over this age.

For diagnosis of IBR , serological tests like ELISA, micro SNT, FAT, indirect HA and CFT can be done by using serum of infected and suspected animals, as well as the isolation , detection of nucleic acid and differentiation of isolates can be done by collecting the nasal swab, vaginal swab, prepucial washing, conjunctival swab, semen sample and milk sample.

The prevention and control of the disease is based on normal hygienic measures taken in farm and herds. Usually a 2-3 week quarantine period is imposed for newly introduced cattle, admitting only seronegative cattle into farm and vaccination of cattle which prevents the development of severe clinical signs and reduce the shedding of virus after infection, but do not prevent infection. Now attenuated and inactivated marker vaccines based on deletion mutants or a subunit of a virion, viz.,glycoprotein gD have been developed. These marker vaccines make possible to distinguish the vaccinated animals from infected ones. By conducting diagnostic test such as ELISA , these vaccine provide the basis for eradication programme(Bosch *et. al.* 1998) .In India live attenuated (Mehrotra *et al.,* 1998) and a beta-propiolactone inactivated BHV-1 vaccine (Mehrotra and Shukla ,1991) were prepared and efficacy was evaluated satisfactorily . However, no vaccine is being used for control of disease in India on large scale.

IBRV is such an entity which has been proved to be a more serious threat to the bovine population in India in terms of various clinical syndromes and association with secondary bacterial infections, latency and poor prevention measures. Scanty literatures are available on seroprevalence and molecular characterization studies of IBR in India as well as in the state of Odisha.

In this context, the seroprevalence studies as well as detection of IBR by polymerase chain reaction (PCR) technique could give a clue about the prevalence of IBR in the crossbred cattle population of Odisha. Keeping the above facts in view this study was under taken with following objectives.

1. Isolation of IBR virus from clinical samples like vaginal swab , semen.
2. Isolation will be attempted using MDBK cell line and will be confirmed by development of characteristic CPE like intraneuclear basophilic inclusions, cowdry type A and serological examination like micro SNT.
3. Details of physicochemical characterization of virion will be carried out .
4. Characterization of IBR virus will be carried out by using purified IBR isolate which will be used in the indirect ELISA for seroprevalence of IBR susceptible cow serum .
5. The purified virus will be subjected to molecular characterization by SDS page restriction endonuclease analysis and using specific set of primer to amplify various gene through PCR reaction.
6. The amplified PCR product will be subjected to further restriction endonuclease analysis.

Chapter-2

Review of Literature

2. REVIEW OF LITERATURE

Respiratory tract, being exposed to atmosphere, is often infected with various viruses and other infectious agents. One such virus is the bovine herpes virus-1 associated with a variety of clinical syndromes affecting both respiratory and genital tracts of bovines in many parts of the world.

2.1 HISTORY AND GLOBAL DISTRIBUTION OF IBR

The BHV-1 is distributed world-wide paralleling the distribution of domestic cattle, but has been eradicated from Austria, Denmark ,Finland, Sweden, Italy,(Province of Bolzano),Switzerland and Norway and control programmes are running in some other countries.(OIE Terrestrial Manual, 2008). It is the one of the emerging disease of cattle resulting from the introduction of cross-breeding programme in the country.

The disease was first described in the1950's in Colorado and California (Schroeder and Moy, 1954; McKercher *et al.* 1955) Before 1955, the disease was known as "red nose" and "necrotic rhinitis". In 1958, Kendrick *et al.*, described its association with infectious pustular vulvovaginitis and latter on with abortion, McKercher and Wada (1964). Of later, the virus has been reported to be associated with infection of respiratory, reproductive, central nervous and enteric systems, and with the eye and skin lesions as well as neonatal infection causing red nose , necrotic rhinitis, pustular vulvovaginitis, balanopothitis, epididymitis, abortion, infertility , in coordination, ataxia, diarrhoea, kerato-cnjuctivitis (Pink eye), ocular carcinoma(eye cancer) ,dermatitis and mastitis (Gibbs & Rweyemamu, 1977; Kahrs, 1977)

2.1.1 In the world

Miller,1955 first reported in U.S.A. as an emerging disease in 1950.Similarly Studdert *et al.* (1961) were first to report on IBR infection from Argentina. From the European continent Lomba *et al.* 1973(Belgium),Fave *et. al.* 1967 (France), Grundar *et al.* 1960(West Germany),Straver *et al.* 1964 (Holland),Moretti *et al.* 1964 (Italy), Steck *et al.* 1969 (Switzerland),

Dawson *et al.* 1962 (United Kingdom) had also reported the occurrence of IBR and IPV/IPB. From African continent Provost *et al.*1964 (Central Africa Republic), Hafez, 1974 (Ezypt) , Mare and Van-Rensburg, 1961 (South Africa) and from Australia Snowdown,1964 were first to report on IBR,IPV IBP infection whereas Bagdadi and Martin,1974 reported the occurrence of IBR and encephalitis from Iran. Webster and Manktelow(1959) and Nishimado *et al.* (1972) also successfully reported the incidence of IBR from Newzealand, Japan respectively.

2.1.2. In India

In India, the disease was first reported as an outbreak of keratoconjuctivitis in cross bred calves (Mehrotra *et al.* 1976). Subsequently isolation of virus from the cases of abortion in cows was also reported (Mehrotra, 1977).Since then the disease has gained high dimensions though out the country. Serological investigations thereafter have indicated the prevellance of IBR infection in alaraming proportion in most of the states of India. The disease was found to be more prevalent in exotic and crossbred cattle than in indigenous breeds. An outbreak of balanoposthitis was reported from A.I. centre in U.P.(Pandey, *et al.* 2000). Similarly the disease has been recorded from Kerala (Sulochana, *et al.*, 1982), Gujurat (Singh, *et al.*,1983) ,Tamilnadu (Manickam and Mohan,1987), Uttar Pradesh(Mehrotra,1977) Orissa (Mishra and Mishra, 1987), Karnatak (Mohan Kumar *et al.*, 1994), West Bengal (Ganguly, *et al.*, 2008) and Andhra Pradesh (Satyanarayana and Suri Babu, 1987b),

2.2 ECONOMIC LOSSES

The disease has been reported not to be highly fatal but it causes considerable economic loss due to abortion, loss of body condition, milk yield, loss of new born calves, temporary failure of conceptions, secondary bacterial pneumonia and cost of treatments.

In fact , the economic importance of BHV-1 infection was realised as far back as the late fifties when a virus variant caused severe respiratory infection in bovines which spread rapidly in feedlots and cattle population.

Wisemann *et al.* (1979) concluded that the losses were due to, firstly,market value of fatal and culled cases , secondly , feeding cost for one to six weeks when fattening cattle did not put on weight and thirdly, treatment cost and value of lost milk production . They also noticed that IBR was of more problem in beef fattening farm than in dairy farm. In large milking herds the losses varies from $ 25 to $ 55 per cow were estimated. (Townley,1971). Shroeder and Moys (1954), estimated heavy losses due to IBR in USA and100% loss due to IBR was reported in Hungery (Bartha,1975). Townley (1975) reported 30% morbidity and a loss of $ 500 in an acute outbreak of respiratory form of IBR in a dairy herd.

Morbidity and mortality rate varies considerably and were lower in dairy herds (8% morbidity and 3% mortality) than in beef cattle, in feedlots in which the mortality rate was usually 20 to 30 percent and rarely up to 100 percent (Barenfus *et al.*, 1963).

Morbidity rate of 90 percent and mortality rate of 30 percent was recorded in an outbreak of nervous form of disease in Australia (Gardiner, 1964). Conception rate fell down from 80 percent to 45 to 50 percent in majority of the cows suffering from herpes vulvovaginitis which were artificially inseminated (Laveso *et al.*, 1984).

2.3. CHARACTERASICS OF BHV-1

2.3.1. Classification

BHV-1 is a member of the genus Varicellovirus in the subfamily Alphaherpesvirinae which belongs to the family Herpesviridae (Roizman and Batterson,1985; Murphy *et al.*1995). It is encpsidated by icosahedral capsid which is surrounded by envelope. The virus measures about 200nm as shown by negative stain preparation containing 162 capsomeres (Watrach and Behnemman, 1966).

BHV-1 isolates can further be differentiated by DNA restriction enzyme analysis into subtypes BHV-1.1, BHV-1.2 and BHV-1.3,(Wyler *et al.* 1989) . BHV-1.1 is associated with IBR and BHV-1.2 is associated with IPV and IPB (Edwards *et al.* 1990) BHV-1.2 isolates can be further differentiated into BHV-1.2a and BHV-1.2b where BHV-1.2a isolate cause abortion and BHV-1.2b is less virulent compared to earlier one.(Metzler,1995).BHV-1.3 which is a neuropathogenic agent, and causes meningoencephalitis and has been newly classified as BHV-5 (Magyar *et al.*, 1993).

2 3.2. Genome

The genome of IBR virus is linear double stranded DNA having a total size of 136-140 kilo base pair(kbp). and comprising a long segments known as unique long sequence(UL) of 104 kbp and a short sequence(Us) of 10 kbp,flanked by internal and terminal inverted repeats (Irs, Trs) of 11kbp each (Roizmann *et al.* 1992). The sequence comprises two genes both duplicated 67 in inverted repeats. Thus BHV -1 encodes at least 69 protiens .(Schwyzer and Ackermann, 1996). The entire gene has been sequenced by international collaboration. And sequencing has mainly done by Schwyzer *et al.* 1996.

2 .3.3.Envelope glycoprotein

The viral glycoprotein located on the surface of virion play an important role in pathogenesis and immunity. Extracellular viral genome codes for about 21 structural polypeptides with molecular weight varying from 31kd to 275kd of which VP1, VP6, VP9, VP10, VP11, VP12, VP13, VP15, VP16, VP17, are glycosylated with molecular weight of 275, 120, 86, 83, 76, 68, 66, 54, 53, and 43kd respectively. (Schwyzer and Ackermann,1996)

It has relatively short reproductive cycle and causes efficient destruction of infected cells .Incubation period is 6-14 days. The virion also posses haemagglutination. activity which is coded by a 90kd glycoprotein (Trepanier *et al.* 1985) The virus entities associated with IBR and that with IPB/IPV are closely antigenically related.

2.4.HOST RANGE AND EXPERIMENTAL INFECTION

The natural hosts for IBR virus were cattle and buffaloes but the outbreaks of the disease have also been recorded in goats (Mohanty *et al.*, 1972), Swine (Darbyshire and Caplan, 1976), mink and ferrets (Porter *et al.*, 1975).

Among the wildlife species, the virus was isolated from prepuce of water buffalo in northern Australia (St. George and Philpott, 1972), pronghorn antelope in USA Hoff *et al.*, 1973).

Serum antibodies were also detected in sheep and goats (small ruminants) by Taylor *et al.*, (1977) and Wafula *et al.* (1985).

Experimental infection has been reported in mule deer (Chow and Davis, 1964), goats (McKercher *et al.*, 1959; Pirak *et al.* 1983; Wafula *et al.*, 1985), ferrets (Smith, 1978), rabbits (Kelly, 1977; Lupton *et al.*, 1980a), skunks (Lupton *et al,* 1980b), sheep (Shankar and Yadav, 1987) and calves/steers (Terpstra, 1979; Noda *et al.*, 1982).

2.5 CLINICAL SYNDROMES OF IBR

Bovine herpes virus – 1 infection in cattle has been associated with respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal disease (Gibbs and Rweyemamu, 1977). A brief description is as follows :

2.5.1. Respiratory form

IBR occurs as a mild and unrecognized disease to an acute form involving the whole respiratory tract with severe inflammation, abundant exudates, foamy salivation and the nasal mucosa which is congested in the early stages turns to yellowish brown diptheritic plaques. There may be rise in temperature, depression, reduced appetite, dullness, coughing, oculonasal discharge and in milking animals, a sudden drop in milk production is commonly observed. (Andrews and Pereira, 1972; Bartha, 1975; Gibbs and Rweyemamu, 1977; Wiseman *et al.*, 1980).

The disease has an incubation period of two to six days depending on dose, route of inoculation and other factors relating to onset of the disease. This form of the disease is commonly observed in feed lots than in diary cattle herd and young ones are highly susceptible to infection (Kahrs, 1977). The disease runs for twelve to fourteen days with high morbidity (30 to 100%) and low mortality (1-10%) in feed lot cattle) (Barenfus *et al.*, 1963).

On postmortem examination, the gross lesions are limited to the muzzle, nasal cavities, pharynx, larynx, trachea and large bronchi. In the upper respiratory tract a variable degree of inflammation is observed (Blood, *et al.*, 1983). Necrotising rhinitis, pharyngitis, laryngotracheobronchitis with extensive pseudomembrane formation and severe pneumonia with or without interstitial emphysema (Allan *et al.*, 1980). The mucosae of bronchi, bronchioles and alveoli were edematous and occasional necrotic foci (Pavlov, 1976). There was congestion and edema of retropharyngeal, bronchial and mediastinal lymphnodes (Rogers *et al.*, 1980).

2.5.2. Genital form- (IPV/IBP

a. In female: The clinical symptom include edematous and hyperaemic vulva with small pustules (1 to 2mm diameter) present all over its surface. These pustules coalesce to form a yellowish white fibrinous membrane which gets detached resulting in ulcers. Lesions usually heal up in ten to fourteen days after the onset of disease (Blood *et al.*, 2000).

Abortions usually occur between fourth and seventh month of gestation (Gibbs and Rweyemamu, 1977; Tanyi *et al.*, 1983); or during the last trimester of pregnancy) (Straub *et al.*, 1982).

Kirkbride *et al.*, (1973) reported that JBHV-1 was the most diagnosed cause of abortion in 16 per cent of 2554 cases in northern plains of USA.

b.In male : Infectious pustular balanoposthitis also develops in one to three days with same type of lesions as in IPV on prepucial mucosa. In uncomplicated cases healing occurs in ten to fourteen days after the onset of disease mild and subclinical form of IPB were also common (Gibbs and Rweyemamu, 1977). Unilateral and bilateral semino S vesiculitis were reported in young bulls (Villar *et al.*, 1987).

2.5.3. Nervous form

The young calves are most commonly affected and show in co-ordination leading to ataxia. Cycles of depression and excitement ending in stumbling and falling with development of clonic spasms of leg, neck and lumbar muscles. Coma and death usually ensue three to four days after onset of symptoms. In some calves, blindness and circling are observed (Weibler *et al.*, 1989).

2.5.4. Alimentary form

Alimentary tract infection is more commonly seen in young calves (Curtis *et al.*,1966). .The major clinical signs include septisaemia and diarrhoea (Rogers *et al.*, 1978).

2.5.5. Neonatal form

Some of the clinical signs recognised are (fever, diarrohea, difficulty in swallowing, focal interstitial pneumonia, respiratory distress, drooling saliva, ocular and nasal discharges , (Blood *et al.*, 2000).

2.5.6. Ocular form

The IBR virus is frequently associated with conjunctivitis (Andrews and Pereira, 1972) where in thin, clear, watery discharge from the eyes which may be so extensive that the hair beneath the eye becomes heavily soiled (Rebhun *et al.* ,1978).

Conjunctiva is hyperaemic and oedematous, in severe cases aversion of eye lids occurs. Secondary bacterial infection is frequent leading to small flecks of pus in lachrymal discharge, keratitis, corneal opacity and corneal ulcerations is also noticed (Gibbs and Rweyemamu, 1977 ; Wiseman *et al.,* 1980.

2.5.7. Dermal form

Dermatitis of teats and udder, and perineal part of body in IBR virus affected animals were noticed (Abraham *et al.*, 1976). The IBR virus was reported to produce ulcerative lesions in mouth and interdigital space of hind limbs. (Dhennin *et al.*, 1979).

2.6. DIAGNOSIS

The different menifestations of BHV- 1 can be diagnosed by clinical symtoms ,physico-chemical properties, biological properties and other tests like serological and molecular methods. IBR is confused with other diseases where respiratory signs are seen, viz,.shipping fever , calf diphtheria, bovine virus diarrhoeal, malignant catarrhal fever, enzootic pneumonia and foot and mouth disease.Simlarly abortion caused by BHV-1 may be confused with bovine epizootic abortion and IPV may be differentiated from contagious granular vulvo vaginitis.

2.6.1. Physico-chemical properties

a. Determination of Nucleic Acid type :

Armstrong *et al.* (1961) observed that bovine kidney cell pre-treated with 5-iodo o 5-fluro deoxyuridine both of which suppressed the growth of IBR/IPV virus while no effect was seen with RNA viruses.

Sabina (1965) while studying the metabolic effect of IUDR on IBR infected bovine kidney cell observed that inhibition of viral CPE could be reversed by thymidine there by implying it to be a DNA virus.

Lillie and Mohanty (1968) observed that IUDR at a concentration of 10AM could completely inhibit the replication of IBR virus after 5 hour of infection in bovine embryonic kidney cell.

Mehrotra *et al.* (1979) while characterizing an IBR isolate also observed the inhibitory effect of IUDR in calf kidney cells and there by proving genome to be of DNA type.

Suribabu and Mallick (1983) while characterizing an isolate of IBR reported that IUDR did not allow the multiplication of IBR virus in primary bovine calf kidney cell and thus proving to be a DNA virus.

Singh *et al.* (1989) used MDBK cell line for primary isolation of IBR virus from nasal swab of cattle and subjected them to IUDR treatment for detailed characterization and found that there was complete inhibition of virus multiplication.

b. Effect of temperature :

Madin *et al.* (1956) isolated an IBR virus from nasal washing of cattle and while subjecting them to thermal stability observed that virus survived at -70°C for 7 months while it remained viable only for 96 hour at 37°C.

Stability of IBR/IPV virus at various temperature have also been documented by Hahnefeld *et al.* (1963) who showed that IBR/IPV virus remained stable for 6 and 2 months at -70°C and -20°C respectively while a substantial reduction in pathogenicity was observed following storage at 5°C for 15 days, besides only remained viable for 24 hours at 37°C. Complete inactivation was seen following exposure at 56°C for 10 minutes for 65°C for 4 minutes.

Snowdown (1964) reported that bovine adrenal gland cell culture propagated IBR virus when exposed at 56°C for 6,12 and 18 minutes a sharp fall of titre 3 log, 4 log and complete inactivation could be found respectively.

While characterizing an isolates of bovine herpesvirus from a leucocyte of calf, Bodon *et al.* (1970) observed that complete inactivation could be observed at 50°C for 15 minutes.

House (1972) while using primary calf testicle, pig kidney, dog kidney and green monkey kidney cell line for the susceptibility of IBR/IPV virus found that all IBR/IPV virus found that all IBR/IPV strains could be only grown in pig kidney cell at 40°C.

Schudel *et al.* (1983) while characterizing an IBR strain propagated in bovine kidney and testis cell culture observed that 90% loss of it's infectivity at 37°C for 15 hour while a more rapid loss of infectivity could be found at 56°C.

Singh *et al.* (1989) while studying the physical properties for an IBR virus strain isolated from aborted cases similarly observed that a complete inactivation could be found following exposure at 56°C for 30 minutes.

c. pH Stability :

While analysing the viability of IBR virus at different pH level from 6 to 9 Griffin *et al.* (1958) observed that the virus reasonably remained stable within that range but looses its activity below pH 6.

Mehrotra *et al.* (1979) while conducting pH sensitive analysis of calf kidney propagated IBR isolate also observed that the virus was sensitive to pH 3.2. A similar observation by Singh *et al.* (1989) was also reported where an IBR virus isolate from an aborted case propagated in MDBK cell line remained sensitive to pH 3.2.

d. Effect of chemical agent :

Griffin *et al.* (1958) found IBR virus was quite sensitive to the action of ether acetone and ethyl alcohol where inactivation of the virus occurred within 24 hours while formalin caused inactivation but at a much slower rate.

Armstrong *et al.* (1961) characterized IBR/IPV virus by treating it with 20% ether overnight and noticed a 100 fold reduction in titre of virus when propagated in calf kidney cell culture.

Rouhandeh *et al.* (1967) reported that an IBR strain having 8 million PFU/ml was completely inactivated with treatment of 50% ether at 37°C while at 0°C approximately 4log virus was found to be inactivated.

Munro and Sabina (1970) studied the inhibitory effect of isatin-β-thiasemicarbazone (IBT) or it's N-methyl derivative on various isolates of IBR virus in calf kidney cell culture and found them all to possess an antiviral effect.

Mehrotra *et al.* (1979) isolated an IBR strain from on aborted case which was found to be sensitive to the action of ether, chloroform and sodium desoxycholate thus proving it to be an enveloped virus.

2.6.2 Biological properties

a. Propagation of IBR virus :

Multiplication of IBR virus have been reported in variety of cell culture systems such as bovine embryonic kidney (Madin *et al.*, 1956; Greig *et al.*, 1958; Gillespie,1959); bovine testis (Bagust, 1972; House, 1972); bovine kidney (Cheatham and Crandall, 1957) which are found to be most susceptible where as ovine kidney, caprine kidney, equine kidney, (Mckercher, 1959), canine kidney, monkey kidney, feline kidney, (Plummer *et al.*, 1969), rabbit kidney, (Mckercher, 1959) were found to be less susceptible. The most widely used cell line is MDBK (Stevens and Groman, 1963), rabbit spleen (Armstrong *et al.*, 1961), bovine turbinate (BT) and embryonic bovine tracheal cell. These cell showed CPE like rounding ballooning with small refractile syncitia and as the CPE progressed cytoplasmic strand linking the adjacent cells are also noticed.

b. Cytopathic effect and inclusion bodies:

Madin *et al.* (1956) observed the CPE with an isolate of IBR recovered from nasal washing in bovine embryonic kidney cell which was characterized by rounding and shrinking of cells with an increased granulation and aggregation of cells in 24 to 28 hours. Such changes continued through 96 hours till majority of cells were affected causing them to be detached form the glass surface.

Cabasso (1957) however could not observe any CPE by IBR virus on HeLa, KB, L cells and chicken embryo fibroblast monolayer.

Mckercher *et al.* (1957) infected bovine embryonic kidney cell with an IBR isolate rcovered from nasal washing and found to show CPE like rounding and granulation of cells which later detached from glass surface.

Derbyshire *et al.* (1962) compared the CPE induced by oxford strain and Colorado strain of IBR in cover slip preparation of bovine kidney cell at 48 and 72 hours post infections which were fixed with Bruin's fluid and stained with hematoxylin and eosin. Both strains produced characteristic

intramuscular eosinophilic inclusion bodies. In a similar study Derbyshire (1963) also observed the CPE induced by an IBR isolate in bovine kidney cell which showed eosinophilic or amphophillic intramuscular inclusion confirming it to be cow dry type A inclusion.

Rouhandeh (1963) observed that no apparent CPE could be observed in primary monkey kidney cell culture by IBR virus up to fourth passage but CPE tends to develop from fourth passage until twenty fifth passages which comprised of aggregation of cells or syncitia and rounding of cells.

Moretti *et al.* (1964) while attempting isolation of a virus in bovine embryonic kidney cell identified it to be an IBR virus basing on its CPE like intranuclear inclusion holies and morphological identification by electron micrography.

In a similar observation Molello (1966) also studied the CPE induced by IBR virus propagated in bovine embryonic cell which included clumping and rounding of cells along with granulation and development of intranuclear inclusion bodies.

Chia and Savan (1974) carried out bovine foetal tracheal organ culture to study the CPE of a field strain of IBR virus which consisted of discrete foci of rounded cells on the epithelial surface which spreaded from cell to cell beneath the ciliated epithelial cell leading to early degeneration of nonciliated cell.

Mehrotra *et al.* (1979) characterized an IBR virus isolated from the aborted cases observed cow dry type A inclusions in calf kidney cover slip preparation.

Al Bena *et al.* (1985) employed secondary calf kidney cell for isolation of an IBR virus from nasal and mouth swab and observed various CPE which included swelling and rounding of cells at 48 hours post infection while following 96 hours post infection all the cells showed rounding and degenerative changes.

Singh *et al.* (1989) observed the CPE of an IBR isolate from a nasal swab in MDBK cell line which consisted of syncitia by about 72 hours post infection while a complete destruction of cell sheet was noticed following 96 to 120 hours post infection. Infected covers lip preparation revealed characteristic intranuclear Cow dry Type A inclusion when stained with hematoxylin and eosin.

Woldehiwet and Rowan (1990) however could not observe any CPE of BHV 1in macrophage cell culture although it supported the viral replication.

2.6.3. Serological technique

a. Enzyme Linked Immuno Sorbent Assay(ELISA) -

It was first described by Engvall and Pearlman (1972). Since then there have been several modifications of these methods. The assays are well suited for the detection of low levels (1.0µg/ml) of antibody . The ability of ELISA to detect BHV-1 antibodies at a much higher end point dilution of serum compared to other serological tests, makes it most useful for routine serological diagnosis as far as sensitivity of the tests are concerned.

Engvall and Pearlman (1972) developed ELISA for the first time which is now being used frequently either for detection of antigen or antibody because of its high sensitivity with various modifications.

Payment *et al.*(1979), carried out indirect ELISA using antigen purified through sucrose density gradient, which gave antibody titre up to 100 times higher than SNT. Of the 250 serum samples screened through ELISA and SNT, 54 serum sample found negative by SNT were positive by indirect ELISA.

Solsona *et al.*, (1980) carried out ELISA in micro plate using crude viral antigen and alkaline phosphatise as an enzyme

Bolton *et al.*, (1981)developed an ELISA for survey of titration of bovine sera for presence of IgG antibodies against IBR virus and subsequently,

Beccaria *et al.*, (1982) have reported rapid ditection of antibodies to BHV-1 by macro and micro ELISA.

Perrin *et al.* (1984) used an ELISA kit to screen both serum and milk antibody against IBR from 16 herds and found an established ELISA threshold OD .0.15 for serum and 0.30 for milk. They also found that a 200 times higher concentration of antibody in serum than milk. Hence negative ELISA results in bulk milk samples cannot exclude the possibility of infection.

Collins *et al.* (1985) while standardizing indirect ELISA for screening of BHV-1 antibody used a single dilutions of serum with both infected cell lysate antigen and purified virus as antigen while comparing the result with CFT and VNT, the CFT assay was enhanced by adding bovine complement and result obtained with single dilution of serum in indirect ELISA was found to be more sensitive than CFT and VNT.

Durham and Sillars (1986) while employing indirect ELISA and SNT for the screening of 2028 serum samples against IBR antibody collected from 166 dairy herds and 172 beef cattle herds, the ELISA gave high level of agreement with SNT in classifying positive and negative samples (98% and 97%) respectively. However 122 serum samples with trace neutralizing antibody was found positive by indirect ELISA.

Edward *et al.* (1986) evaluated the sensitivity of 5 serological tests for the detection of IBR antibodies from 300 bovine sera using indirect ELISA for IgC and IgM,IHA and two methods of VNT and found that sensitivity of ELISA and 24 hours neutralization tests were similar and superior to IHA and 1hour neutralization test.

Riegale *et al.* (1987) used competitive ELISA for detection of BHV -1 antibody using a bovine antibody and virus neutralizing monoclonal antibody. The assay showed improved sensitivity over VNT and an enhanced VNT where antibody virus mixture were incubated for 24 hours. Antibody could be detected earlier after infection by ELISA.

Nakajima *et al.* (1989) developed an ELISA technique using purified glycoprotein from IBR infected cell lysate and observed it to be more sensitive and specific in detecting BHV type-1 antibody. The ELISA test was found to be more sensitive and specific than SNT.

Florent and Wiseman (1990) used a capture ELISA for the detection of IgM antibody to IBR and BRSV using monoclonal antibody to bovine IgM and the second monoclonal antibody for antiviral antibody. Result showed that virus infection can be confirmed 5 to 10 days after appearance of clinical sign with serum samples collected from both experimentally and naturally infected animal.

Ungar and Abraham (1991) in a similar observation an IgM indirect ELISA with two calves infected with BHV-I up to 60 days, where IgM antibody could be detected 6th day post infection while increased IgG antibody could be found 9th day post inoculation whereas SNT antibody could be detected only on 13th day post infection thus proving the sensitivity of IgM ELISA over IgG ELISA and SNT.

Durham *et al.* (1991) carried out serological serological survey with 283 bull calves serum where samples were collected on days 0, 14, 28 and 70 against IBR, PI-3, BRSV and BVD where an incidence rate of 20, 81, 32 and 21% were found respectively by indirect ELISA. However animal vaccinated with IBR, BRSV and PI-3 vaccine showed higher prevalence of antibody.

Darcel (1992) compared the results of indirect ELISA and SNT for the detection of IBR antibody with 1065 serum samples from A.I center and found that 129 ELISA positive serum samples only 18 were found to be positive by SNT and from the 111 ELISA false positive serum samples 22 were shown to be nonspecific binding of immunoglobulin to polystyrene of the ELISA plate.

Dereget *et al.*(1993)compared the efficacy of two type of SNT and ELIS A for detection of IBR antibody from 100 serum samples and concluded that 32% were positive by both type of SNT while ELISA could detect more positive reactors which may be due to false negative result.

Pandit and Srivastava (1993) carried serological survey against IBR with 94 serum samples from aborted cows and 135 inconstant cattle serum using indirect ELISA kit (Flow laboratory diagnostic kit, U.S.A) and found an incidence rate of 73.40% and 37.78% respectively in addition to that while indigenous crossbred, exotic cattle and buffaloes showed an incidence rate of 55.50, 76.70 and 50.50% respectively with history of abortion, while the in contact animals showed an incidence rate of 40, 36, 66.70 and 30% respectively.

Mohan Kumar *et al.* (1994) used commercial IBR ELISA kit to screen 10 serum samples from Karnataka where they recorded an incidence rate of 40%.

Pandit and srivastava (1995) comparatively observed the efficacy of dot ELISA and plate ELISA with 239 bovine serum samples form Haryana against IBR antibody where an seroincidence rate of 51.9% could be detected by plate ELISA where as 48.5% could be detected by dot ELISA.

Renukaradhya *et al.* (1996) employed avidin-biotin ELISA for the serological survey of IBR in cattle and buffalo in 3 southern state of India, where they could detect an overall prevalence of 50.9% in cattle and 52.5% in buffaloes. However breeding bulls revealed higher incidence rate of 95% from Tamilnadu 41.4% from Karnataka while 55.4% incidence of IBR could be observed from aborted cows.

Suresh *et al.* (1999) employed avidin-biotin ELISA for the detection of IBR antibody to screen 2473 cattle serum samples collected from 18 states and could found an incidence rate of 49.21% with highest incidence rate of 100, 96.55 and 95.35% for Orissa, Andaman and Nicobar Island and Jammu and Kashmir respectively. Similarly IBR incidence rate with 955 serum samples from buffalo was found to be 9.01%.

Abd El-moniem *et al.* (2006) observed that ELISA is more.sensitive than agar gel precipitation test (AGPT) and virusneutralization test (VN).

Mahmoud *et al.*,(2009),isolated and identified Infectious bovine rhinotracheitis-Infectious pustular vulvovaginitis (IBR/IPV) virus from a herd of cattle and buffaloes, suffering from respiratory and genital disorders by conducting ELISA test.The percent of positive reactors in buffaloes were is less than percent of cattle present in contact with them in the same open farm. ELISA is considered the most rapid, reliable,

Suribabu *et al.* (1984 .) did not find any antigenic differences between Indian , European and American isolates of IBR virus by ELISA technique. In another work, Suribabu *et al.*, (1984) reported that ELISA is 32 times more sensitive than conventional indirect haemagglutination test in detecting IE<R antibodies in bovine serum samples.

b. Fluorescent Antibody Technique (FAT)

Fluorescent antibody technique has been successfully employed by several workers in the identification of IBR virus in cell culture as well as naturally infected animals.

Bergmann and Hahnefled (1967) using both direct and indirect immunofluorescence technique detected first virus specific fluorescence in the nuclear membrane and later in the cytoplasm of infected cell cultures.

Soos and Halasz(1976). Stated that the fluorescence was demonstrated not only from cytoplasm but also from nuclei of IBR virus infected cells. Soos and Halasz,(1976).

Majewska *et al.*, (1984) were of the opinion that indirect immunofluorescence with fluorescein labelled antibovine gamma globulin was twice as sensitive as direct FAT for BHV.Immunofluorescence was considered as a rapid diagnostic test for detection of virus infected epithelial cells and smears from cases of respiratory diseases, abortions (Nettleton, 1986; Edwards *et al.*, 1988).

Immunofluorescence, histological methods and virus isolation were found to be 100 per cent, 87 per cent and 44 per cent efficacious in the diagnosis of IBR .

c. Indirect Haemaglutination (IHA)

Dean and Burgess (1976) detected 97.5 per cent positive samples with IHA titres more than two fold and concluded that IHA1 was more sensitive than SNT. Dannacher *et al.* (1979) were of the opinion that PHA gives a quick and easy titration of antibodies to IBR and was suitable for routine testing in district laboratories.

In another survey, conducted by Satyanarayana and Suribabu (1987) in Andhra Pradesh, 77.46 per cent of 914 serum samples from bovine population tested positive for IBR antibodies by IHA Test.

A micro IHA was successfully used by Suribabu *et al.*, (1984) for antigenic analysis of Indian, European and American isolates of IBR virus.

Kashoek *et al.* (1994) carried out an alternative method of virus identification is by direct demonstration of BHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (16)

Mahmoud *et al.* (2009) identified the BHV-1 by dispersing trypsinized MDBK infected with the isolates on microscopic slide and fixing with chilled acetone and applied Fluorescent Antibody Technique.

d.Serum Neutralisation Test (SNT)

Afshar (1965) and Frank *et al.* (1975) suggested that the demonstration of significant rise in neutralising antibody titres could be of diagnostic value in IBR. Saxegard (1968) used serum neutralisation test (SNT) to detect subclinical infections in bulls with IPB virus infection.

Dunne *et al.* (1973) reported that 1:6 SN titre was considered as positive for IBR/IPV infection in both the sera of foetus as well as adult animals.

Lum and Reed (1986) were of the opinion that four virus specified glycopeptides with molecular weights of 69-75 K, 77-81 K,81-92 K and 108 - 115 K daltons were appeared to be involved in inducing SN antibodies as detected by SDS - PAGE and western blotting.

Suribabu *et al.* (1984) used SNT as one of the tests to establish the antigenic relationship of Indian isolates of IBR/IPV virus employing European and American IBR virus strains. They reported that all the strains tested were antigenically related.

e. Agar gel immunodiffusion test (AGID)

A gel diffusion test was used for detecting antibodies against IBR/ IPV virus in Kenya (Kaminjolo and Gicho, 1972).

Le Jeune *et al.* (1977) developed a micro immuno diffusion test for detecting antibodies to IBR in bovine sera using antigen prepared from IBR virus infected MDBK cell culture. The results of the test obtained at 48 hours correlated well with those of SNT.

Abd El-moniem *et al.*(2006) observed that ELISA is more.sensitive than agar gel precipitation test (AGPT) and virus neutralization test (VN).

f. Complement fixation test (CFT)

CFT, when applied on IBR serum samples the titres were low, the test was found to be of not much value (Rossi and Kiesel, 1974). The CF assay was enhanced by adding bovine complement (Collins *et al.*, 1985). Karadzhov and Khristova (1980) used CFT for diagnosis of BHV-1 infection.

g. Immunoperoxidase test (IPT)

Smith *et al.* (1987) concluded that IPT was more sensitive than FAT for detecting IBR virus and bovine respiratory syncitial virus. IPT using monoclonal antibodies was specific and sensitive compared with viral isolation for diagnosis of BHV-1 encephalitis (Rodriguez *et al.*, 1989).

2.7.2 MOLECULAR METHOD

Recent attention in diagnostic virology has been directed towards the development of nucleic acid techniques for the detection of virus in clinical specimens. Nucleic acid hybridization and polymerase chain reaction (PCR) were developed as ideal diagnostic tools for the detection ofBHV-1 in clinical specimens because their rapidity, sensitivity and specificity.

Several hybridization formats such as dot-blot hybridization (Vilcek *et al.*, 1993a and 1993b), *in situ* hybridization and Southern blot hybridization (Kibenge *et al.*, 1994 and Xia *et al.*, 1995) with radioisotope (Kibenge *et al.* 1994; Xia *et al.*, 1995), labelled probes have been applied for the detection of BHV-1 in nasal swabs and semen. PCR with Southern blot hybridization has been developed as a diagnostic in which 0.01 TCID50/100µl of BHV-1 could be detected in 1:20 diluted bovine semen (Kibenge *et al.*, 1994 and Xia *et al.*, 1995).

a. DNA extraction

Engelenburg *et al.* (1993) developed a PCR assay to detect BHV-1 in bovine semen using a purification method that eliminated interfering components. This was acheived by separating the semen into a seminal fluid, a non sperm cell and a sperm head fraction and preparing seminal lysates from these fractions using sodium-N-laurylsarcosine, proteinase K and sheared salmon.

Wiedmann *et al.* (1993) used chelating resin Chelex 100 to prepare viral DNA from artificially inoculated samples of extended and raw semen for use in the PCR assay.

Engelenburg *et al.* (1995) lysed the seminal fluid at 60°C for 60 minutes using NaCl, Tris, EDTA, Tween 20, proteinase K and bacteriophage λ DNA. The DNA present in the lysate was directly purified by chromatography.

Xia *et al.* (1995) pelleted the virus by ultracentrifugation at 134000g for 4 hours at 4°C in SW28 rotor and resuspended in TE buffer prior to SDS and proteinase K digestion for 18 hours.

Marsi *et al.* (1996) used Non-idet P-40 (NP-40) and proteinase K for extraction of DNA of BHV-1 from semen sample. Wagter *et al.* (1996) gave a brief treatment with proteinase K to lyse free virus, virus present in non-sperm cells and virus adhering to the spermatozoa. Genomic bovine DNA was not released by this treatment.

Tiwari *et al.* (2000) reported PCR based detection of BHV-1 from infected cell culture supernatant simply by heating in boiling water bath without further extraction or purification of DNA and its comparison with PCR on viral DNA purified by SDS-proteinase K lysis followed by phenol: chloroform extraction and precipitation by absolute ethanol.

Deka *et al.* (2005) used spermatozoa-free supernatant for DNA extraction which was obtained by mixing and centrifuging the semen with an equal volume of maintenance medium. This spermatozoa-free supernatant was then subjected to proteinase K treatment and phenol:chloroform extraction.

B. Polymerase Chain Reaction (PCR)

The PCR technique was discovered in April 1985 by Kary Mullis (Mullis, 1990). The basic principle of PCR is amplification of the template (target) DNA. This is achieved by the use of synthetic oligonucleotides that correspond to sequences within the target and which is a thermostable DNA polymerase. The exponential increase in target is achieved by subsequent rounds of denaturation, primer annealing and extension by DNA polymerase. After the amplification of DNA, the reaction is assayed for the specific product in agarose gel with ethidium bromide staining and also by hybridization with a cloned probe or oligonucleotide probe, or by the digestion with a restriction enzyme. The widespread success of PCR as a technique comes from the fact that it is rapid, automated, efficient, sensitive and specific.

Various PCR assay for the ditection of BHV-1 have been described using the primer of gB gene Vilcek,1993),gC gene (Van Engelenburg *et al.*,1995) , gDgene (Wiedmann *et al.* 1993; Gee *et al.* 1996 and the thymidine kinase (tK) gene of BHV-1 (Kirbenge *et al.* 1994) with variable sensitivities . Another nested PCR was developed for the detection of BHV-1 in bovine semen (Masri *et al.,* 1996). This assay could detect BHV-1 DNA in semen at 0.25-2.5 TCID50.

Vilcek (1993) described amplification of the 468 bp fragment of the BHV-1 genome by PCR using gI gene .For successful amplification the thermal denaturation (100°C/8 min, ice) of the DNA sample was carried out

prior to the cycling (95°C for 1 min, 56°C for 1 min, 73°C for 1 min). The presence of glycerol markedly enhances the PCR.

Kibenge *et al.* (1994) 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. 0.01 TCID50/100µl of BHV-1 could be detected in 1:20 diluted bovine semen by using this method.

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, tracheal mucosa) collected from 16 different outbreaks in Scotland, 18 were found positive by PCR and 13 by virus isolation. Some samples of isolated DNA 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 BHV-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.

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 BHV-1. The primer pairs were used for amplifying genomic DNA of BHV-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 BHV-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. Of a total of 162 ejaculates, 51 were found positive by virus isolation, whereas PCR detected BHV-1 DNA in 73.

Fuchs *et al.* (1999) detected BHV-1 in whole-blood samples derived from naturally infected cattle. Sensitive PCR assays specific for glycoprotein B (gB), gC and gE of BHV-1 allowed the detection of one BHV-1 DNA copy in 105 to 107 peripheral blood leukocytes.

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

Rola (2002) collected 149 semen samples every 3-4 days interval up to 107 days post infection (dpi) and analysed them with the virus isolation test and PCR assays. He treated the bulls with dexamethasone from 44 to 48 dpi to reactivate the latent infection. He found only 9 (6%) semen samples collected between the 2nd and the 9th dpi reacting positively in the virus isolation test.

Using PCR technique and primers for gC and gD glycoprotein genes of BHV-1, he detected the presence of the virus in 11 (7.4%) and 16 (10.7%) samples of semen, respectively. More positive results of amplification were obtained with nested PCR in which he found 44 (29.5%) positive samples.

Deka *et al.* (2005) screened 51 serum samples from apparently healthy breeding bulls for BHV-1 A-B ELISA assay, revealing a seroprevalence rate of 45.09%. Out of 24 semen samples (12 from sero-positive and 12 from sero-negative bulls), the PCR detected BHV-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 2 tissue samples in dairy cattle of Puawy by means of virus isolation and PCR assays. They found 24 positive sera for IBR antibody while virus was isolated from only one nasal swab and one tissue sample PCR with external primers detected the presence of BHV-1 in 11 nasal swabs and in one tissue sample.

Gupta *et al.* (2006) carried out a rapid and sensitive PCR based assay for BHV-1 in semen using primers designed from g C gene utilizing glass milk for sample preparation.

Marsi et al. (1996) amplified viral thymidine kinase (tk) gene fragment and found a 202 bp band within 514 to 716 bp of BHV-1 tK gene confirming the specificity of the assay.

c. Restriction Enzyme Analysis (R.E.A.)

Ross *et al.* (1999) partially sequenced the glycoprotein B (gB) and D (gD) genes from five ruminant alphaherpesviruses, bovine herpesvirus 1(BHV-1), bovine herpesvirus 5 (BHV-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%, and a more variable gD gene, with homologies ranging between 71.3 and 98.9%.On the basis of sequence data for the gB gene, a nested PCR combined with restriction enzyme analysis (REA) of the PCR products was developed for the simultaneous detection and identification of the viruses that were studied.

Gupta. *et al.* (2006) carried out the PCR based rapid and sensitive test for detection of BHV-1 by using primer designed for glycoprotein C of 520 bp and the amplified 520 bp product was characterized using restriction endonuclease and Southern bllot hybridization. The sensitivity of detection was as low as 10^{-1} plaque forming unit (pfu) BHV-1.

D'Arce *et al.*(2002) carried out restriction endonuclease analysis and monoclonal antibody (MAb) analysis on 12 Brazilian isolates and three reference strains of Bovine herpes viruses. Viral DNA was cleaved with BamHI, BstEI, EcoRI, HindIII and PstI. The monoclonal antibody panel allowed the differentiation between types 1and 5 viruses.

Chapter-3

Materials and Methods

3. MATERIALS AND METHODS

3.1. COLLECTION OF SAMPLES

a. Vaginal swab- Twenty two vaginal swabs were collected from state dairy farm Khapuria and from field which had history of repeat breeding ,abortion and infectious postular vulvovaginitis (IPV) case . Vaginal swabs were collected aseptically and transported in HBSS in ice. (Table-1)

b. Semen sample- Five frozen semen samples consisting of Jersey,Cross-bred Jersey and Red Sindhi were collected aseptically in HBSS and transported in ice.(Table-2)

c. Serum samples – All together 165 blood sample were collected and serum sample were separated aseptically and kept in Mc Carteny's vial with addition of sodium azide . The serum sample were transported in ice. The serums were collected from cows with history of repeat breeding ,abortion and infectious postular vulvovaginitis cases . All sera samples were heat-inactivated at 56^0 C for 30 minutes and stored at -20^0C until tested.(Table-3)

3.2. ISOLATION OF IBR VIRUS

a. Cell line- MDBK (Madin Darbey Bovine Kidney) cell line was obtained from CADARD unit, IVRI ,Izatnagar, U.P.

The vaginal swabs and semen samples were syringe filtered (milipore, 0.45µm) and subjected to log dilution at 10^{-1}. MDBK cells were grown and subcultured with Eagle's minimum essential medium (EMEM, Sigma) with 10% fetal bovine serum in tissue culture flasks (Nunlon, 25cm^2) having cell concentration of 2×10^5/ml. Following development of complete monolayer after 3 days of incubation at 37°C, the cells were ready for infection. The confluently grown MDBK cell monolayers were washed three times with maintenance media (MEM) and diluted samples were inoculated and kept at 37°C in CO_2 incubator for 1hour for viral adsorption. After 1hour of adsorption the cells were washed thrice with sterile maintenance medium (MM) to remove unbound virus particles and replaced with maintenance media.

Table-1. DETAILS OF VAGINAL SAMPLE S

Sl. No.	Name of the farm/Clinic	No. of samples	Animal
1.	LBD farm,Khapuria	12	CBJ cow
2.	TVCC,OVC,Bhubaneswar	10	CBJ cow
Total	-	22	-

Table-2. DETAILS OF SEMEN SAMPLES

Sl. No.	Name of the farm/Clinic	No. of samples	Animal
1.	TVCC,OVC,Bhubaneswar	5	CBJ,JERSEY , RED SINDHI

Table-3. DETAILS OF SERUM SAMPLES

Serial Number	Name of the district	CBJ	Indigenous	Total
1	Rayagada	95	5	100
2	Kalahandi	10	-	10
3	Balasore	18	-	18
4	Cuttack	27	-	27
5	Khurda	10	-	10
Total		160	5	165

Cultures were incubated at 37°C to observe cytopathic effect for 5 days. Daily observation was carried out at 48hour, 72hour, 96hour and 120hours for development of CPE. Likewise three blind passages of ivaginal and semen samples in MDBK cells were carried out. With each passage after complete development of CPE the bottles with monolayer cultures were subjected to 3 cycles of alternate freezing and thawing and then the cell culture supernatant was harvested aseptically in sterile Mc Carteny's vial. These infected cell culture supernatants were inoculated into Leighton's tube with cover slip having MDBK cell grown confluently for detailed study of the microscopic changes.

These infected cover slip preparation and suitable control cover slip preparation were subjected to fixation with 10% formal saline solution at 72hours, 96hours and 120hours interval and then stained with Haematoxylin Eosin method (HE method). The stained coverslip preparation was DPX mounted on slide for study of detailed microscopic changes.

3.3 TITRATION OF THE VIRUS

Following the development of characteristic CPE the virus samples were subjected to titration in MDBK cell grown in 96 well flat bottomed tissue culture plates with cover by the method of Reed and Muench (1938) and The tissue culture infective dose ($TCID_{50}$) was calculated. Following titration, micro serum neutralisation test was carried out with reference IBRpositive sera which was supplied by kind courtesy of Dr.S.K.Samal.Vice Dean, School of veterinary Medicine , Maryland,USA. After observing positive neutralisation, means inhibition of cytopathic effect. Isolates were serologically identified as IBR strain. These isolates were further preserved and kept at -20^{0}c for study of detailed physico-chemical characteristics

3.4. PHYSICO-CHEMICAL PROPERTIES

3.4.1.Nucleic acid type determination

Metabolic inhibitor like 5-iodo-2-deoxyuridine (IUDR) 10^{-4}M) (Koch Light Laboratories Limited, Colnbrok-Bucks, England) was used according to the Armstrong *et al.* (1961) in the present study for the determination of

nucleic acid type. A stock solution of 1mg/ml of IUDR was prepared with triple distilled water pH 7.4 and added to the MM so as to attain a concentration of 50 µgm of IUDR/ml. Two sets of test tubes 15 in each sets with confluently growth MDBK cell monolayers were washed with sterile MM and about 1ml of MM containing IUDR was poured to one set of the test tubes while the other sets served as control (without IUDR only MM). After 1 hour of incubation at 37°C, media from both the sets of test tubes were discarded and inoculated with log diluted stock virus inoculum (ranging from 10^{-1} to 10^{-5}) in sterile MM. three tissue culture tube, each was infected with single dilution (0.1 ml/tube) and after 1 hour of adsorption at 37°C, the monolayers were washed with MM and IUDR treated tubes were replaced with MM containing IUDR while the control tube receive a MM without IUDR. The infected cell culture tubes were incubated at 37°C for 4 to 5 days and $TCID_{50}$ was determined in MDBK cells grown in microtitre plate.

3.4.2.Thermostability

The method of Snowdown *et al.* (1964) was used to study the thermostability of 3rd passage level of IBR inoculum. For this, MDBK cells were grown to confluent monolayer in test tubes and infected with a 10^{-2} dilution of the stock 3rd passaged IBR virus and observed for development of CPE for 4 to 5 days. Following complete development of CPE 3 sets of infected test tubes were subjected to 3 cycles of alternative freezing and thawing and stored at -20°c. The harvested virus was subjected to titration and for this the inoculum was subjected to log dilution in mm. MDBK cells were grown to confluent monolayers in 96 well flat bottomed tissue culture Corning plate. Two rows of six wells were infected with log diluted inoculum ranging from 10^{-1} to 10^{-6} with 100 µl/well in quantity, following 1 hour of adsorption, the inoculum was discarded and the wells were replaced with MM. The plates were sealed properly and kept inside a desiccators with 5% Co_2 tension and incubated at 37°C. Suitable virus control and cell controls were also kept for comparison. Following 4 to 5 days of incubation at 37°c, the plates were observed for development of CPE and $TCID_{50}$ was calculated as per the method of Reed and Muench (1938).

To carry out the thermostability at various temperatures, one set of each harvested inoculum was subjected to temperature at 41°C, 50° Cand 55°C for 30 minutes in a water bath and transferred immediately to an ice bath. All these 3 sets of tubes were subjected to titration with MDBK cells grown in microtitre plates in confluent monolayer and $TCID_{50}$ was calculated as per the method mentioned earlier.

3.4.3. Cationic stabilisation

Stability of MDBK propagated IBR virus in presence of cations was studied according to the method of Tschider *et al.* (1974). Briefly, IBR infected cell culture supernatant was subjected to treatment of 50°C and 56°C at 1 hour in presence of 1M $Mgcl_2$ solution and immediately it was transferred to an ice bath, suitable control i.e. virus without the presence of $Mgcl_2$ was also subjected to same treatment and finally both treated and untreated virus samples were subjected to titration in MDBK cell line grown in microtitre plate.

3.4.4. Stability to PH

Virus stability at varying pH was studied as per the method of Griffin *et al.* (1958). 3 sets of test tube containing 0.2 ml of harvested inoculum were treated with 2.2 and 1.6 ml of sterile citrate phosphate buffer, to bring the pH 4.5 and 6 while the third set inoculum was treated with IM $NaHco_2$ solution to make the pH 8.2. Control inoculum received only MM. All these set of viral inoculum were incubated at 37°C for 30 minutes and $TCID_{50}$ was determined in cells grown in microtitre plate.

3.4.5. Effect of chloroform

Action of lipid solvents like chloroform on IBR virus was studied according to the method of Hahnefeld *et al.* (1963). Harvested cell culture supernatant was treated with 10% v/v analytical grade chloroform and shaken for 10 minutes, it was further kept for 1 hour at 4°C and centrifuged at 1000 rpm for 5 minutes. The aqueous lower layer was carefully removed with help of a sterile pasteur pipette and both treated and untreated control were subjected to $TCID_{50}$ determination for assessing the viability of virus in MDBK cell line grown in microtitre plate.

3.4.6. Action of formalin

Effect of formalin on IBR virus was studied according to the method of Straub (1965). Briefly, infected cell culture tubes were treated with 0.3% formalin analytical grade and the mixture was incubated at 37°C for 18 hour. The residual infectivity was titrated in MDBK cell line and untreated virus served as control.

3.5. THE BULK PRODUCTION OF IBR VIRUS

Following third passage of IBR virus in MDBK cell and their identification by micro SNT, bulk production of IBR virus was attempted to be used in ELISA and molecular characterization. For this MDBK cells were grown in Roux flask and confluent monolayers were infected with third passage isolated IBR virus. Following 96 hours of infection when more than 90% cells showed CPE the virus was harvested after three cycles of freezing and thawing and in this way five litres of infected supernatant were collected and subjected to centrifugation at 10000 rpm for 30 min in refrigerator centrifuge. The supernatant were aseptically collected in sterilized flask and treated with PEG-6000 @ 6% and kept in magnetic stirrer for 30 min for concentration of the virus. The PEG treated cell culture supernatant were subjected to centrifugation at 10000rpm for 30 min in a refrigerator centrifuge (Bakeman). The supernatants were decanted and pelleted concentrated virus was collected by flushing with 2ml of TRIS EDTA buffer (pH 8.0). The aliquots were kept at -20°C.

3.6. PURIFICATION OF IBR VIRUS

The concentrated IBR virus was subjected to purification in an ultra centrifuge (Sorval-65). The ultra centrifuge tubes were first layered with 35% w/v sucrose suspension and the concentrated virus were layered over it and subjected to the centrifugation at 8000g for 6 hours. The pelleted virus samples were collected and cooled. A discontinuous sucrose density gradient was prepared with 35% sucrose solution and 65% sucrose solution. The 65% sucrose solution was first loaded into the ultra centrifuge tube and above it the 35% sucrose solution was layered carefully to avoid mixing. The pelleted

virus was layered over the two gradients and subjected to ultracentrifugation at 8000g for 12 hours. Following ultracentrifugation the purified virus was collected with help of a pateur pipette by siphoning method and collected in a sterile vial with addition of two ml of fresh EDTA buffer. Purity of purified virus was checked in a spectro photometer by taking two OD values, i.e. OD at 260nm and OD at 280nm. The ratio of OD was found to be 1.2 there by confirming purity of virus.

3.7. INDIRECT ELISA

Indirect ELISA was conducted as per methodology of Suresh *et. al.* (1999). The purified virus was subjected to protein estimation by Lauries method .It was found to be 28.6 mg/ml. Following the protein estimation of purified virus, the virus sample was diluted in carbonate bicarbonate buffer having pH 9.2, so as to attain a concentration of five micro gram per ml. The diluted virus sample in carbonate bicarbonate buffer were used as coating antigen and similarly normal MDBK cell lysate following protein estimation was used as negative antigen in indirect ELISA.

a.Test proper

1. The ELISA plates were kept at 4°C over night following each wells were coated with hundred micro litre of diluted virus in carbonate bicarbonate buffer.
2. Next day the plates were emptied and washed thrice with PBS Tween 20 (washing buffer pH 7.4)
3. The wells were blocked with blocking buffer and plates were kept at 37°C in an incubator for half an hour.
4. For test proper kits developed by Animal Disease Monitoring and Surveillance unit, Hebbal, Bangalore was used.
5. Following blocking the wells were washed five times with washing buffer and serum samples were subjected to dilution in PBS Tween 20 at 1:100 dilution and hundred micro litre was used in each well and subjected to incubation at 37° c for one hour.

6. Following one hour incubation the wells were emptied and washed five timeswith washing buffer. Then hundred micro litre of biotin anti IgG conjugate (1:30,000 dilution) was added to each well and incubated at 37° c for one hour.

7. Well were emptied and washed thrice with washing buffer and hundred micro litter of Avidin HRPO conjugate (1:15,000 dilution) was dispensed into each wells and plates were incubated at 37° c for twenty minutes

8. Following incubation the wells were emptied and washed with washed buffer and hundred micro littre of chromogen-substrate solution was added to these wells and incubated for8-10 minutes at 37° c

9. Soon after colour development fifty micro litre of stopping solution was added into each wells and OD was recorded in an ELISA Reader (Multi scan USA) at 492 nm.

b. Interpretation

P.P.(%) =Average OD of the sample / Median OD of strong positive serum X 100

P.P. at 45 = Sample is positive

P.P. at < 45 = Sample is negative

3.8. MOLECULAR CHARACTERISATION BY PCR

3.8.1. Extraction of viral DNA

For extraction of viral DNA from purified virus ,kits developed by QIAGEN was used.

1. Twenty micro litre of QIAGEN proteinase in buffer AW1 and AW2 was dispensed into a 1.5 ml micro litre microcentrifuse tube.

2. Two hundred micro litre of purified virus sample was added to the tube.

3. Two hundred micro litre of AL buffer was added to the tube and mixed by pulse-vortexing for fifteen seconds

4. The mixture was incubated at 56°C for10 minutes

5. The tube was centrifused to remove drops from the inside of the lid.

6. Ethanol (96-100 %) was added to it in two hundred micro litre quantity and again it was subjected to pulse-vertexing for 15 seconds

7. The mixture was added to QIAamp mini spin column in 2 ml collection tube and centrifused at eight thousand rpm for one minutes.

8. The QIAamp spin column was kept in a two ml collection tube and the filtrate is discarded from the tube.

9. The QIAamp spin column was opened and 500 ml of buffer AW-1 were added to it and centrifuged @ 8000 rpm for one minute, following which filtrate is discarded.

10. The QIAamp mini spin column was added with 500 micro litre of AW-2 buffer centrifuged at 15,000 rpm for 3 minutes.

11. The QIAamp mini spin column was kept in a 1.5 ml microcentrifuge tube and filtrate was discarded from collection tube. The mini spin column was treated with two hundred micro litre AE buffer and incubated at room temperature for one minute and then centrifuged 8000 rpm for one minute.

12. The extracted DNA was kept in TRIS- EDTA buffer in 200 micro litre in a microcentrifuge tube.

3.8.2. Polymerase chain reaction-

The forward and reverse primers of gB, gC and gD gene regions were taken which are 443 bp, 173bp and 343 bp respectivily

a.Preparation of reaction mixture in PCR tube.

i. 10x buffer -	2.5 µl
ii.Magnesium chloride (25 mM) -	2.5 µl
iii.dNTP mix. (10 mM) -	1 µl
iv. Primer - 1 -	1µl
v.Primer - II -	1µl
vi.Enzyme (Taq polymerase) (3U /µl) -	1µl
vii. Template (DNA) -	5 µl
viii.Distilled water --	11µl
	----------------------
	25 µl

b. Conditions

PCR tubes were put in thermal cycler programmed with following cycling conditions.

Initial heating at 95° c for 15 minutes (initial denaturation) were followed by 30 cycle of following steps.

Denaturation ---	94 °c	50 sec
Annealing ---	58 °c	50 sec
Extention----	72 °c	50 sec

Total 35 cycles were followed by

Final extention --	72° c	5 minutes

Following the completion of conditions, the sample tubes were taken out and checked for specific amplified products .

c. Gel electrophoresis

Confirmation of amplified products by Agarose gel electrophoresis was done

i. Agarose gel(1.0 %) was prepared containing ethidium bromide to a final concentration of 0.5 µg / ml in mini gel tray of sub marine gel electrophoresis system in 1X TAE buffer, pH 8.3.

ii. The wells were loaded with amplified products along with 100 bp ladder marker in adjacent well and operated the gel at 80 v for 2 hours. Dye was added to each sample

iii. The gel was taken out from the apparatus and seen under the UV transilluminator.

iv. The presence of bands of 443 bp, 173 bp and 343 bp in length in test samples were checked for gB, gC and gD gene rigion amplification respectively.

v. In negative sample there was no amplification of DNA and no visualisation of specific amplification.

3.8.3. Restriction endonuclease analysis of PCR products

Following agarose gel electrophoresis of PCR products the purity was checked as per their base pair size.

a. Gel extraction (As per QIAquick Gel Extraction Protocol)

1. The DNA fragment in the agarose gel was subjected to extraction first with clean , sharp scalpel
2. The gel slice was kept in a colour less tube and weight was taken to which three volume of buffer QG was added to one volume of gel.
3. The mixture was incubated at 50° c for ten minutes and until the gel slice was completely dissolved. Simultaneously the tube was subjected to vertexing for 2-3 minutes for complete dissolve of the gel.
4. After the gel slice had dissolved completely and colour of the mixture was turned up to yellowish colour.
5. One gel volume of the isopropanol was added to the mixture and it was placed in QIAquick spin column in 2 ml collection tube .
6. The tube was centrifuged for 1 minute after discarding the QIAquick column was kept in same collection tube .
7. To wash, 0.75 ml of buffer P was added to QIAquick column and centrifuged for 1 minute. The suspension was discarded and QIAquick column was centrifuged for 1 minute at 13000 rpm following which the column was kept in sa 1.5 microcentrifuge tube .

8. For elusion of DNA ,50 µl of buffer EB r (10 mM TRIS Cl , pH-8.5) or water to the centre of the QIAquick membrane and centrifused for 1 minute.

b. Restriction endonuclease analysis

The PCR product gC - 173bp, gB - 443bp and gD - 343bp were subjected to restriction endonuclease analysis with Hae- iii, Hind-i and Nor-i enzymes with the following protocol

1. For gCgene,173 bp DNA sample

Enzyme Hae-iii	1 µl
Buffer	1µl
Template (PCR product)	8 µl

With final volume of 10 µl which was incubated for 1 hour at 37 °c.

2. Like wise PCR product gB-443 bp with

Enzyme Hind-i	1 µl
Buffer	1 µl
Template PCR product	8 µl

Were processed as mentioned above

3. In similar fashion for PCR products for gD DNA (343 BP) was subjected to digestion with ,

Enzyme Nor-i	1 µl
Buffer	1 µl
Template (PCR product)	8 µl

and was subjected to incubation at 37 °c for 1 hour.

Following the digestion, the digested product were screened agarose electrophoresis.

Chapter-4

Results

4. RESULTS

4.1. ISOLATION OF IBR VIRUS

Twenty two vaginal swab and five frozen semen sample in HBSS which was subjected to membrane filtration were used for IBR virus isolation in MDBK cell. Confluently grown MDBK cell in tissue culture flasks were infected with 10^{-1} diluted samples in MEM maintenance media and incubated at 37^0 c and daily observation was carried out at 24, 48, 72, 96 and 120 hours post infection period for development of CPE. After two such blind passages, three more passages were carried out in MDBK cells grown in tissue culture flask and cover slip preparation in Leighton's tube following 48 hours of infection. Few cells were found to be CPE like granulation of cells and rounding. at 72 hours post infection more cells were found to be rounded and there was development of syncitia. As the incubation period progresses to 96 and 120 hours respectively, more than 90% of cells revealed intense CPE like rounding , syncitia and lysis of the cells. Similarly cover slip preparation at 72 hours and 96 hours were fixed with 10 % formal saline solution and stained with Haematoxylin and Eosin method .The stain preparation revealed characteristic CPE like rounding , granulation of cells, syncitia development and characteristic cowdry type A intranuclear basophilic inclusion. (Fig.-2). There are also development of intracytoplasmic vacuolation and intra nuclear vacuolation. Controlled cover slip preparation did not revealed any CPE.(Fig-1)

4.2. SEROLOGICAL IDENTIFICATION OF IBR ISOLATE

Following the TCID 50 calculation of IBR cell in MDBK cell which revealed attire of $10^{5.6}$/ml, 100 $TCID_{50}$ of which was used in micro serum neutralisation test using a positive reference sera sample in micro SNT. After 72 hours all the isolates were confirmed to be IBR by observing completly neutralisation of cytopathic effect at a serum dilution of 1:32, there by confirming the isolates to be IBR strain.

4.3. PHYSICO CHEMICAL CHARACTERASTICS OF IBR ISOLATES

4.3.1. Nucleic acid type determination

In presence of 50 µm/ml of IUDR no CPE could be observed for IBR virus, while the control inoculums showed characteristic CPE and had original inoculums $TCID_{50}$ .There by indicating the nucleic acid of IBR virus to be DNA.

4.3.2. Thermostability

MDBK propagated third passaged reference IBR virus having a $TCID_{50}$ of $10^{5.6}$/0.1 ml was subjected to temperature treatment at 45^0C, 50^0C and 56°C for 30 minutes in a water bath and transferred to an ice bath immediately. All the heat treated inoculums were subjected to titration for the determination of $TCID_{50}$ in MDBK cell grown in micro titre plate. Result of the $TCID_{50}$ showed that viruses heat treated at 45°C had a $TCID_{50}$ of $10^{3.8}/^{0.1}$ thereby showing a decrease near 2 log, but virus treated at 50°C showed a $TCID_{5C}$ of 10' 70.1 ml. However virus treated at 56°C at 30 minutes appeared to completely inactivated the virus as no CPE could be found.

4.3.3. Cationic stabilisation

Stock virus inoculum of IBR treated with 1M $MgCl_2$ and held at 50° and 56°C for 30 minutes. This two samples when titrated in MDBK cells revealed a $TCID_{50}$ of $10^{5.6}$/0.1 ml in both the treated samples. The results indicated that IBR virus is stabilized by 1M $MgCl_2$.

4.3.4. Stability of pH

Stock virus inoculums treated with sterile citrate buffer in varying amount and 1M $NaHCo_3$ solution to bring the inoculum pH 4.5, 6 and 8.2 were all subjected to incubation at 37°c for 30 minutes. Such treated samples were subjected to $TCID_{50}$ determination which revealed that IBR virus at pH 4.5 had 3 $TCID_{50}$ of $10^{2.8}$/o.1ml there by showing an /almost a decrease of 3 log where as at pH 6 and 8.2, $TCID_{50}$ was found to be $10^{5.6}$/0.1 ml for both the sample. The results clearly indicated that virus is labile at pH 4.5 while it was stable at pH 6 and 8.2.

4.3.5. Effect of chloroform

MDBK propagated stock IBR virus treated with 10% v/v chloroform when titrated revealed no CPE while control inoculums had the same $TCID_{50}$ of $10^{5.6}$/0.1 ml. This clearly indicated that virus is completely inactivated in presence of lipid solvent like chloroform, there by proving it to be an enveloped virus.

4.3.6. Effect of formalin

In presence of 0.3% formalin virus appeared to be completely ' inactivated as no CPE could be observed.

4.4. INDIRECT ELISA

All together 165 serum sample collected from cows and bulls having history of repeat breeding ,abortion and bulls having history of IBP were screened by indirect ELISA with the kits developed at Project Director,Animal Disease Monitoring and Surveillance Unit, Hebbal, Bangalore. Following standardisation with positive antigen, negative antigen, positive serum and negative serum where cut of value was of OD were determined. The P P % which is more than 45 and above were declared as positive .In this way 33% of serum sample were found positive by Indirect ELISA for IBR serosurveillance study. (Fig. -3 Pie Diagram ,4,5).

4.5. POLYMERASE CHAIN REACTION

Three sets of primer gC (173 bp), gB (443 bp) and gD (343 bp) corresponding to the giycoprotein gene of IBR virus were used in the present study to amplify the gene product against template DNAof isolated virus. Crrespondingly PCR products of 173 bp,443 bp and343 bp could be observed after electrophoresis, thereby confirming the isolated viral DNA to be of IBR origin (Fig. -6, 7)

4.6. RESTRICTION ENDONUCLEASE ANALYSIS

Three sets of endonuclease enzyme like *Hae*-iii,*Hind* –iii and *not*-i were used in the present study to know the specific PCR product against gC

gene (173 bp), gB gene (443 bp) and gD gene (343 bp). Following restriction endonuclease analysis ,the products after agarosegel erlectrophoresis revealed DNA fragments of 121 bp ,52 bp for gC (173 bp) product while against gB (443 bp) product, the size of the fragment obtained were 229 bp & 241 bp. In similar fashion against gB (443 bp), the size of the DNA fragment obtained were 145 bp & 198 bp (Fig. 8 ,).

PLATE NO. - I

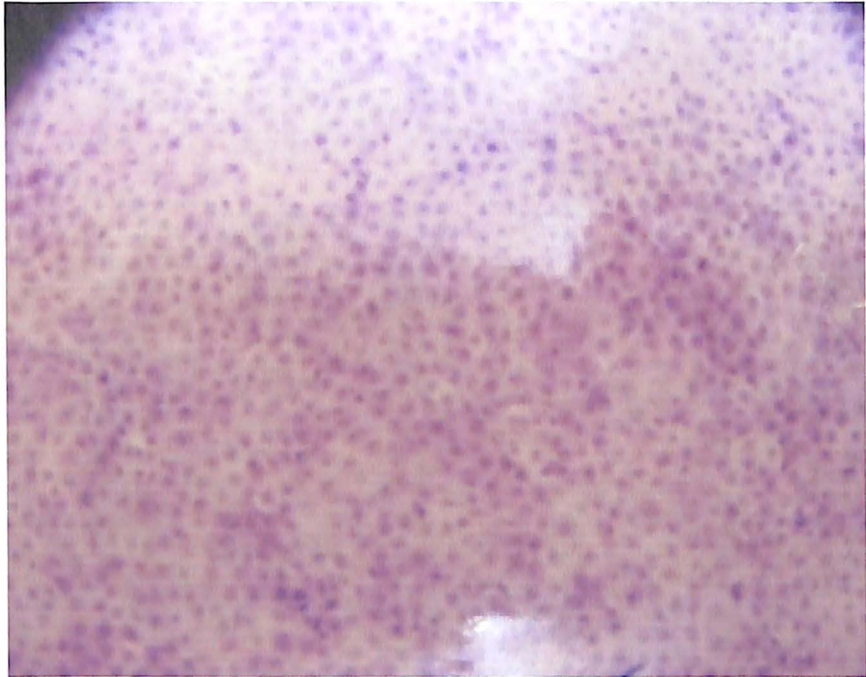


Fig. no. 1: Normal healthy MDBK cell line stained with HE stain

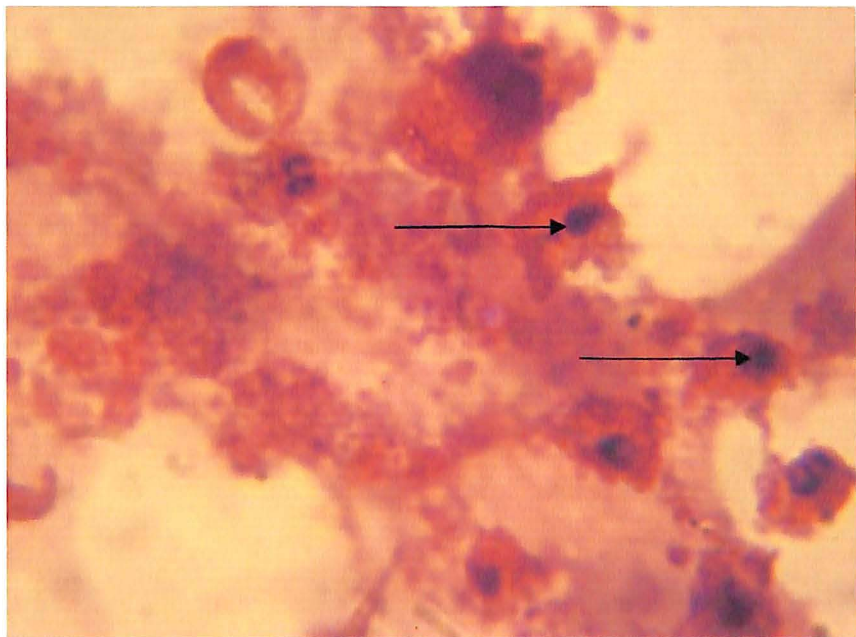


Fig. no. 2: MDBK cell line showing CPE (Cell lysis, Intra-nuclear inclusion bodies)

PLATE NO. - II

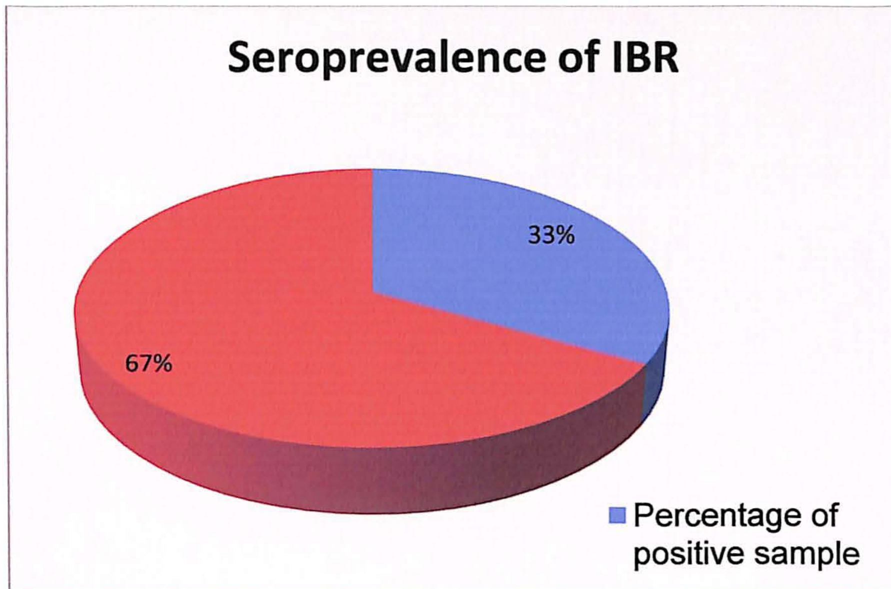


Fig. no. 3

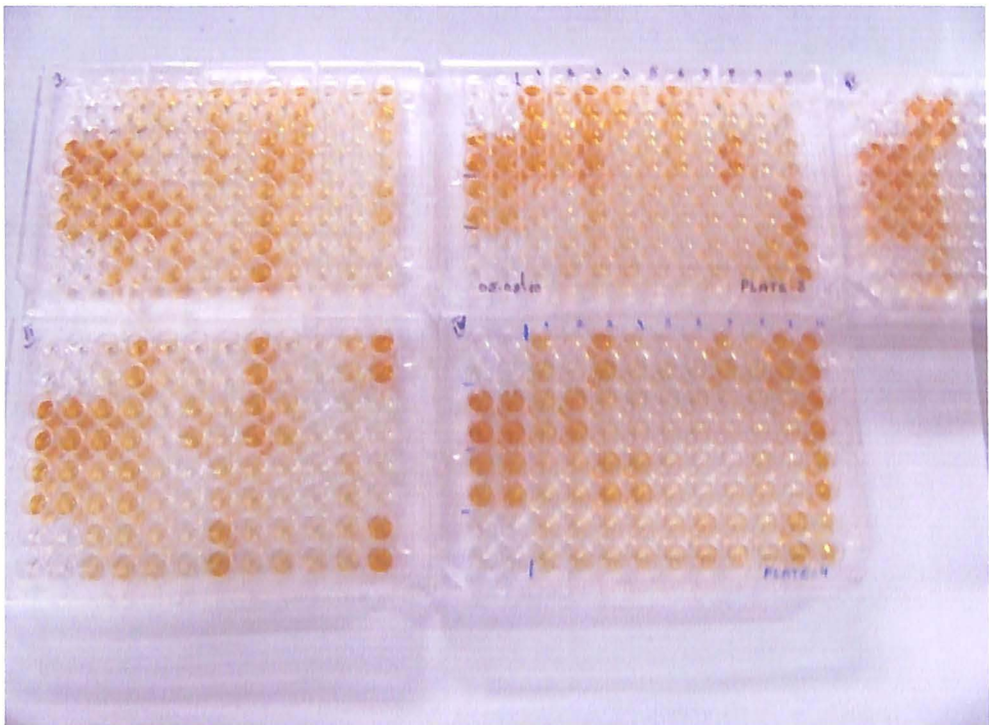
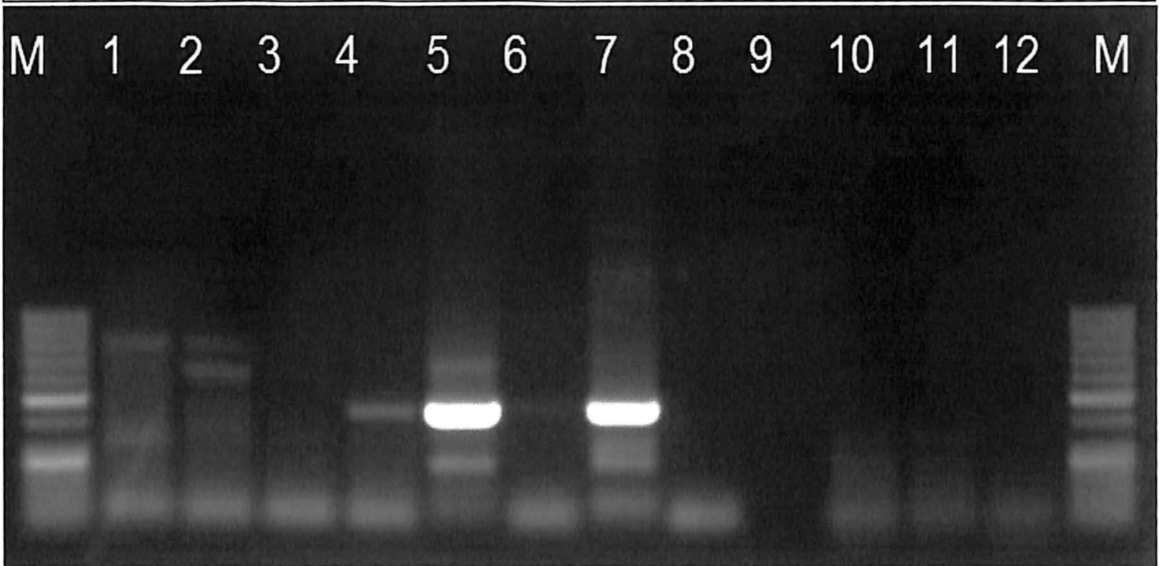


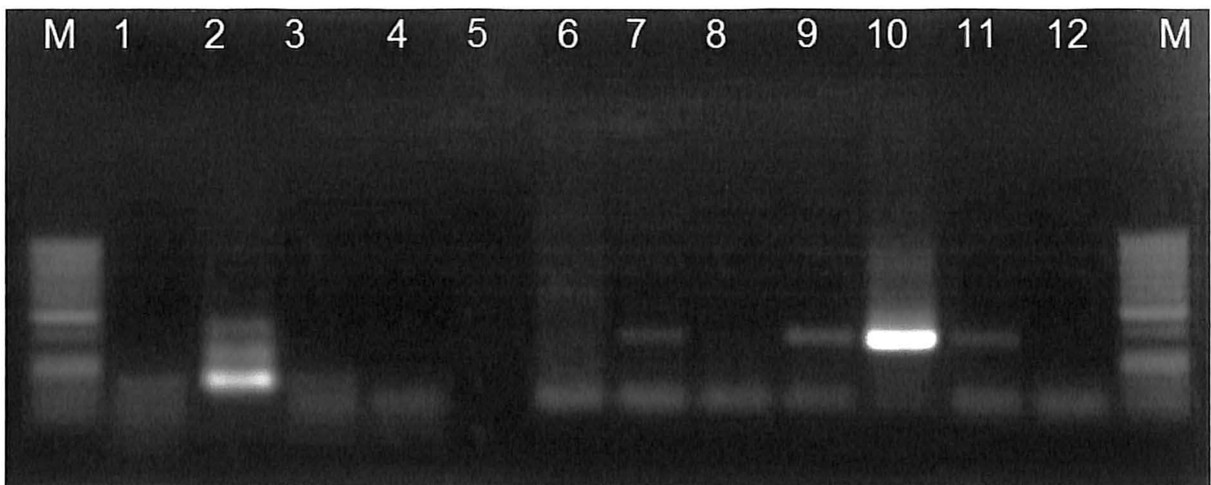
Fig. no. 4: A-B ELISA (165 samples)

PLATE NO. - IV

Fig. no. 6: PCR test for gC, gB & gD gene of BHV - 1

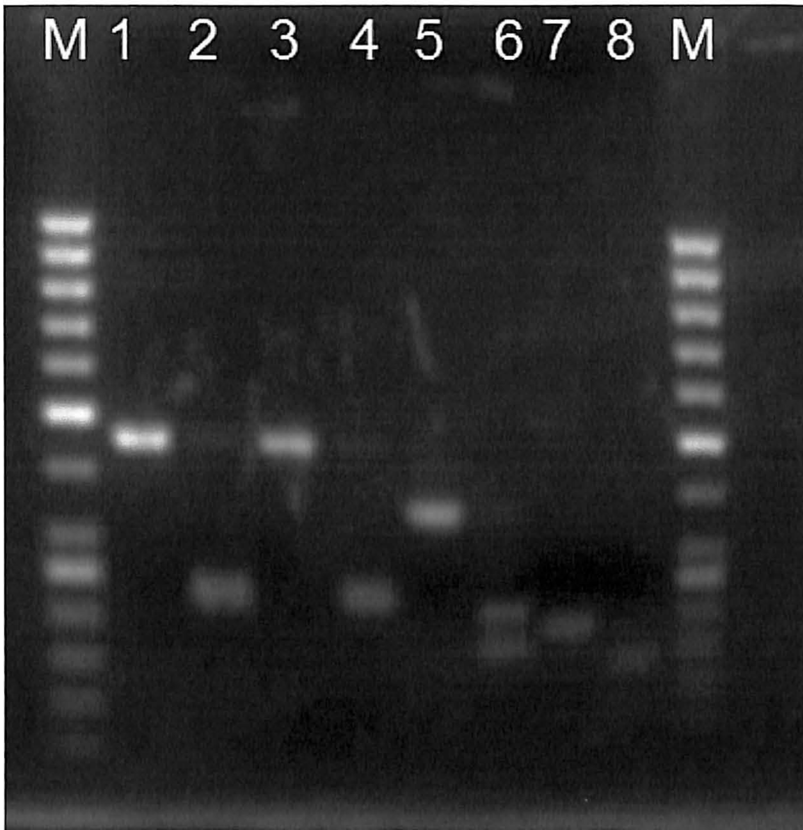


Lane M_50bp ladder
Lane 1-gB(443)-
Lane 2-gB(443)-
Lane 3-gB(443)-
Lane 4-gB(443)-
Lane 5-gB(443)-positive control of Department
Lane 6-gB(443)-
Lane 7-gB(443) PD_ADMAS positive control
Lane 8- gB(443)negative control
Lane 9-gC(173) Negative control
Lane 10-gC(173)-
Lane 11-gC(173)-
Lane 12-gC(173)-



Lane M_50bp ladder(SM1133)
Lane 1-**gC(173)**-
Lane 2-**gC(173)**- **positive control of Department**
Lane 3-**gC(173)**-
Lane 4-gC(173)- Negative control
Lane 5-GAP
Lane 6-gD(343)
Lane 7-gD(343)
Lane 8- gB(443)
Lane 9-gD(343)
Lane 10-gD(343)- **positive control of Department**
Lane 11-gD(343) -
Lane 12-gD(343) -negative control

PLATE NO. - V



Lane M : 50 bp molecular size marker
Lane 1 : 443 bp amplicon of gB region (443F/R)
Lane 2 : *HinfI* digestion of the 443 bp amplicon yielding 229 bp and 214 bp fragments
Lane 3 : 443 bp amplicon of gB region (443F/R)
Lane 4 : *HinfI* digestion of the 443 bp amplicon yielding 229 bp and 214 bp fragments
Lane 5 : 343 bp amplicon of gD region (gD F2/R2)
Lane 6 : *NarI* digestion of the 343 bp amplicon yielding 145 bp and 198 bp fragments
Lane 7 : 173 bp amplicon of gC region (gC F1/R1)
Lane 8 : *HaeIII* digestion of the 173 bp amplicon yielding 52 bp and 121 bp fragments

Fig. no. 7: Restriction endo-nuclease analysis of PCR products for gC, gB, gD gene

Chapter-5

Discussion

5. DISCUSSION

Infectious Bovine Rhinotracheitis (BHV-1) is an acute contagious respiratory and genital infection which is also associated with other clinical syndromes like meningo- encephalitis, conjunctivitis, arthritis and mastitis (Mc Kercher ,1965). The first report of the disease was traced in Colorado state of U.S.A. in 1955 and thereafter the disease was reported from all most all countries. Because of development of latency, repeat breeding, abortion are frequent features of genital infection and there is no authentic vaccine for control of the programme and incredible economic loss are encounter (Darcel and Dorwood, 1972)

Diagnosis of the disease chiefly based on typical clinical symptoms, isolation of virus and various serological tests by micro SNT, FAT and similarly seroprevalence study is generally carried out in tests like IHA, CFT,micro SNT and sandwitch ELISA and monoclonal antibody based competitive ELISA (Darcel, 1992, Penn *et al.*, 1990 and Maglione *et al.*,1992).

Since reports are forth coming on use of molecular biological technique like application of restriction endonuclease analysis, PCR and sequencing of IBR viral DNA. The diagnosis of IBR infection has become more sensitive and specific in addition to various functional role of IBR gene. (D'Arce *et al.*, 2002).

In the present study attempt has been made to isolate IBR (BHV-1) strains from clinical samples like vaginal swab and semen samples from animals with history of repeat breeding, abortion and infectious balano-posthitis (IBP).The materials were collected in HBSS and subjected to syringe filtration. For isolation of IBR strain MDBK cells were grown in tissue culture flask and cover slip preparation in Leighton's tube with cell concentration of 2 x 10^5/ml using MEM (Sigma) with 10 % FCS. The monolayers were infected with 10^{-1} diluted sample and after one hour adsorption, the cell sheet was washed thrice with MEM (Maintenance media) and replaced with MEM. After 24 hours of incubation few cells were found to be rounded whereas after 42 hours more number of cells were found to be rounded and after 72 hours cells showed granulation, increased number of

rounded cells and syncitia development at 96 hours more than 90 % cell showed CPE with intense syncitia development following two blind passages and 3 passages were given to viral sample in MDBK cell. Simultaneously the cover slip preparations were also infected and at 48 hours, 72 hours and 96 hours, the cover slip preparations were fixed with 10 & formalin saline solution stained by H& E methods. Un affected control cells were simultaneously processed .The stain preparation revealed characteristic CPE like rounding of cells with intense stain, syncitia development, cytoplasmic and nuclear vaculation and characteristic Cowdry type A intranuclear basophilic inclusions.

Such CPE has already been reported by Cheatham and Crandel, 1957, using calf kidney cells. and Singh *et al.* (1989) by using MDBK cells. which correlates with our findings.

Preliminary identification was carried out in present study by using serological tests like micro SNT , using a known positive IBR reference sera , which revealed a complete inhibition of CPE with titre 1:32, there by confirming it to be IBR isolate. In this way altogether 5 isolates of IBR strains were identified to be IBR strains. Use of micro SNT has already been reported by Fedida *et al.* (1982) Collins *et al.* (1985) Singh *et al.* (1989)

Following identification of IBR strains by micro SNT detailed characterisation was attempted based on physicochemical properties

Preliminary identification of virus warrants systematic procedures like physico-chemical characterization, in the present study Detailed physico-chemical properties of reference IBR virus was studied in MDBK cell line. The results of exposing the IBR virus to different temperature revealed that the viruses heat treated at 45°C had a $TCID_{50}$ of $10^{3.8}$/0.1 ml as compared to the original inoculums having titre of 10^{56}/0.1 ml, which nearly snowed a fall of log 2 titre. Further the residual virus titre treated at 50°C had a $TCID_{50}$ of $10^{1.2}$/0.1 ml. while virus treated at 56°C for 30 minutes failed to reveal any CPE suggesting a complete inactivation of virus. Similar findings were also reported by Griffin *et al.* (1958); Hahnefeld *et al.* (1963); Bagust , (1972); Mehrotra *et al.*, (1979. loc.cit) and Singh *et al.*

(1989). However, in a retrospective study Griffin *et al.* (1958. loc.cit) could observe complete inactivation of IBR/IPV virus as early as 21 minutes at 56°c. Stabilization of IBR virus at various molar concentration of divalent cations is of immense importance in preservation of virus and vaccine preparation, in the present study MDBK propagated third passaged reference IBR strain when treated with IM $MgCl_2$ did not reveal any fall of titre either at 50°C or at 56°c for 30 minute indicating the virus to be stabilized with the agents. Such observation was recorded by Stevens and Croman (1963. loc.cit); Tschider *et al.* (1974); Mehrotra *et al.* (1979.loc.cit) and Singh *et al.* (1989.loc.cit) when treated the virus with 0.5M $MgCl_2$. Sensitivity of IBR strain to varying PH revealed that there was a substantial drop of virus titre pH 4.5 while it remained stable at pH-6 and pH-8.2. Similar finding has also been made by Griffin *et al.* (1958.loc. cit), Hahnefeld *et al.* (1963); Mehrotra *et al.* (1979.loc.cit) and Singh *et al.* (1989.loc.cit). Effect of various chemicals like Chloroform and formalin on IBR virus viability in this study revealed that chloroform (10% v/v) and formalin (0.3%) completely inactivated the virus. A similar finding has also been reported by Straub (1965); Rouhanden (1967); George and Philpott (1972), Suribabau and Mallick (1983) and Singh *et al.* (1989.loc.cit).

Determination of nucleic acid type using IUDR revealed the virus nucleic acid to be DNA which was also used by Persechino and Orfei (1955); Sabina (1965); Alvellini *et al.* (1967) and Mehrotra *et al.* (1976.loc.cit) for Determination of nucleic acid type. The results of various physico-chemical parameters of the reference IBR virus in the present study appeared to be in par with the findings of other workers.

For serosurveillanance study and molecular characterisation of IBR viral DNA ,bulk production of IBR strain in MDBK cell was attempted and $TCID_{50}$ following 3rd passage was determined and it was found to be $10^{-5.6}$. The bulk production of MDBK cell was carried out using Roux flask and 5 litres of infected cell culture supernatant was concentrated by using PEG 6000 at 6 % concentration and then subjected to purification of sucrose density gradient .The purified virus was stored in presence of TRIS-EDTA buffer. Part of the purified virus was subjected to protein estimation , which

was found to be28.6 mg /ml by Lauries method. Following protein estimation , the purified virus was diluted in coating buffer so as to attain a concentration of 5 µg / ml , which was used to coat the wells of the ELISA plate in 100 µl / well. Similarly normal MDBK cell lysate were used as negative antigen. Indirect ELISA was carried out with the kit developed at P.D. ADMAS ,Hebbal, Bangalore with 165 serum samples collected from cows and bulls with history of repeat breeding , abortion .IPV/IBP using a cut value. The PP was standardised and any serum sample with more than 45 were considered as positive. In this way all together 33 % serum sample were found to be positive for IBR antibodies by indirect ELISA .The A-B ELISA has already been conducted by Renukaradhya *et al.* (1996); Suresh *et al.* (1999) and Rajesh *et al.*(2003), whose findings correlates to our findings.

With the recent trend in molecular biological technique like restriction endonuclease analysis of genomic DNA ,PCR amplified cDNA proof sequencing and cloning, it has become more authentic to study IBR viral DNA at genome level with precise nature of the sensitivity and specificity over the conventional serological test for diagnosis of IBR infection . In the present study all five strains purified IBR were subjected to extraction of viral DNA. For this QIAGEN kit 3 sets of primer like gC (173 bp), gB (443 bp) and gD (343 bp) were used to ampify PCR product against the template DNA by conventional pcr method. After PCR amplification the products were screened by agarose gel electrophoresis and amplified DNA fragments were found to be 173 bp ,443 bp and 343 bp respectively. Such amplification of target DNA were sequenced by PCR method (Rola *et al.* (2002) has also observed that similar size of PCR amplification product by using primer for gC and gD glycoprotein of BHV-1.Similarly Mahmoud *et al.*(2009); Gupta *et al.* (2006) have carried out PCR tests to detect BHV-1.

Therefore it indicates our finding are in accordance of finding of other workers

After amplification of PCR, the products were subjected to restriction enzyme analysis using *Hae*-iii for gC (173 bp), *Hind*-iii ifor gB (443 bp) and enzyme *Nor*-i for gD (343 bp), which resulted in generation of DNA

fragments at 121 bp, 52 bp and 229 bp, 214 bp and 145 bp, 191 bp respectively .Such restriction endonuclease analysis have been reported by Gupta. *et al.* (2006) had used gC gene primer(520 bp) for detection of BHV-1 and PCR product was further characterized by restriction endonuclease analysis. Similarly Lyaku *et al.* (1996) ;Ross *et al.* (1999) has carried out the PCR and Restriction endonuclease analysis

The restriction endoneuclease analysis in present study simulates the finding by other workers ,there by proving the sensitivity and specificity of genomic analysis of IBR PCR products.

In addition to the conventional serological tests like CFT, IHA, FAT, m-SNT and indirect ELISA, more attempt should be made by using sandwich ELISA and polyclonal antibody or monoclonal antibody, and competitive ELISA by using monoclonal antibody for processing diagnosis for IBR seroprevalence study . Likewise , detection of antigen directly from cell culture supernatant or infected materials should be attempted by using monoclonal antibody western blot analysis.

Following PCR amplification and after identification of gene encoding for glycoprotein, attempts should be made to clone the genes in vaccinia virus vector for development of recombinant of vaccine, which is free for hassels possessed by intact virion live attenuated vaccine as they go for a latent infection resulting a production of a fully virulent virus particle and natural host as they possess Latency Associated Gene (LAG).

Chapter-6

Summary

6. SUMMARY

1. An attempt has been made in present work to isolate IBR strain from clinical samples like vaginal swab and semen having history of repeat breeding and abortion, in MDBK cell.

2. All together five semen sample and 22 vaginal swab were used for isolation of IBR strain in MDBK cell and resulted in isolation of five strains which are finally identified by using micro SNT.

3. Biological characterisation of identified IBR strains, revealed, characteristic CPE like granulation of cell rounding, syncitia, lyses and intra nuclear cowdry type A basophilic inclusion

4. Physico -chemical characteristic revealed the nucleic acid with DNA through IUDR treatment, sensitivity to ether , complete inactivation at 56^0C for 30 minutes, stabilisation with 1M magnesium chloride , labile at pH4.5 & stable at pH6-8.2 ,complete inactivation with 10 % v/v chloroform and 0.3 % formalin at 18 hours incubation.

5. Following the successive passage of virus in MDBK cell, all the five strains revealed a TCID50 of 106.5 which was used for bulk production of virus.

6. Following bulk production of virus, it was subjected to concentration by treating with PEG 6000 at 6% concentration which was purified by sucrose density gradient, ultracentrifugation. The protein concentration was found to be 28.6 mg/ml which was used for coating the ELISA plate and molecular characterisation by PCR.

7. Indirect ELISA was carried out using a ELISA kits developed by Project Director, Animal Disease Monitoring and Surveillance Unit, Hebbal, Bangalore with 165 serum samples collected from cows and bulls with genital disorder like abortion, repeat breeding granular vulvovaginitis and infectious balanoposthitis (IBP) which revealed 33% of the sample positive for IBR antibodies

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8. The purified virus DNA was extracted with a kit by QIAGEN which was used to amplify the target DNA using 3 sets of primer gC (173 bp), gB (443 bp) and gD (343 bp) which resulted in same base pair product.

9. PCR amplified product was subjected to restriction enzyme analysis by using Hae –III,Hind-III and Nor-I for gC (173 bp), gB (443 bp) and gD (343 bp) which resulted in yield of 121 bp &52 bp, 229 bp & 214 bp and145 bp &195 bp respectively.

Chapter-7

Conclusion

7. CONCLUSION

Isolation of IBR strain from clinical sample like semen, vaginal swab of animals having genital disorder like abortion , repeat breeding granulo vulvovaginitis, infectious postular vulvovaginitis and infectious balano posthitis were attempted in present study by using MDBK cell line. All the five isolated revealed characteristic CPE like granulation, rounding of cell ,syncitia, intra cytoplasmic and intra nuclear vacculation had characteristic intra nuclear cowdry type A basophilic inclusion.

Following third passage the virus strain was identified by micro SNT referring to positive serum . All the five isolates following serological identification were subjected to detail physico chemical characterisation which proved that nucleic acid to be of DNA type ,the virus labile at acidic PH 6-8.2 and stabilised in presence of 1M magnesium chloride, completly inactivated by both 56^0c for 20 minutes and 3% formalin and inactivated with treatment of chloroform The ultracentrifuge purified virus was as coating antigen in indirect ELISA. And 165 serum sample was screened through indirect ELISA revealed a 33% . In view of such prevalence rate, regular screening should be carried out either by indirect ELISA or by monoclonal antibody sandwitch ELISA, so that these infected animals should be segregated and not to be used for breeding programme. Attempt should be made to use more improvised serological test like competitive ELISA with help of monoclonal antibody as these test are more sensitive and specific.

Three sets of primer which were used to amplify the purified target DNA by the use of 3 sets of primers like gC (173 bp), gB (443 bp) and gD (343 bp) resulted in similar amplified product and these product was further characterised by using 3 sets of restriction endonuclease enzyme like *Hae*-III,*Hind*-III and *Nor* –I resulted in production of DNA fragments 121 bp &52 bp, and 229 bp &214 bp and145 bp &198 bp .Since all these primers are specific for envelope glycoprotein.

The amplified product should be cloned to produce a recombinant DNA vaccine. Besides more attempt should be made for sequencing of different gene encoding for protective antigen, which can be used in recombinant DNA technique for production of an authentic recombinant DNA vaccine. Alternatively cDNA prove prepared by PCR method can be used to detect virus specific DNA directly from clinical sample through *in situ* hybridisation test.

Chapter-8

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Appendix

APPENDIX

1. Phosphate buffer saline (PBS, pH-7.2)

Ingredients	**gm/l**
Sodium chloride	8.00gm
Disodium hydrogen phosphate	1.15gm
Potassium chloride	0.20gm
Potassium dihydrogen phosphate	0.20gm
Tripled distilled water	1000ml

The pH of the solution was adjusted to 7.2 and autoclaved at 121°C and 15lb pressure for 15 minutes.

Cell culture media and solution:

1. Maintenance media:

Ingredients	**gm/l**
Eagle's MEM (Sigma)	9.4
Tripple distilled water	1000ml

The final pH was adjusted to 7.2

2. Growth media:

Maintenance media	90ml
Fetal bovine serum	10ml

3. Trypsin-versene solution

Ingredients	gm/l
NaCl	8.00gm
KCl	0.400gm
KH_2PO_4	0.200gm
Na_2HPO_4	1.900gm
$NaHCO_3$	1.00gm
D-Glucose	0.500gm
Trypsin (1:250)	1.700gm
EDTA	1.400gm
Phenol red	1ml
Distilled water	to make 1000ml

Ingredients were mixed by stirring on magnetic stirrer for 2 hours and sterilized by membrane filtration.

Reagents for ELISA

1. Coating buffer (carbonate bicarbonate buffer pH 9.6)

Ingredients	gm/l
Sodium carbonate	0.318gm
Sodium bicarbonate	0.586gm
Sodium azide	0.040gm

The volume was made upto 200ml with distilled water.

2. Washing buffer (PBS Tween 20 pH 7.4)

Ingredients	gm/l
Sodium chloride	8.0gm
Potassium chloride	0.200gm
Disodium hydrogen orthophosphate	1.445gm
Potassium dihydrogen phosphate	0.200gm
Sodium azide	0.200gm
Tween 20	0.500ml
Distilled water	1000ml

3. Blocking buffer

PBS (pH 7.2)	100ml
Bovine gelatin	1.00gm
Tween 20	0.05ml