DETECTION OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS BY IMMUNOFLUORESCENCE AND POLYMERASE CHAIN REACTION

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DECLARATION

I hereby declare that this thesis, entitled **"DETECTION OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS BY IMMUNOFLUORESCENCE AND POLYMERASE CHAIN REACTION"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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Anupama K.

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Introduction

1. INTRODUCTION

The productivity and performance of animals is mainly based on their health status. India has a huge cattle and buffaloe population frequently exposed to several diseases. Among the viral diseases, Infectious Bovine Rhinotracheitis (IBR), caused by Bovine Herpes Virus-1 (BHV-1) occupies a key position. Considering the socio-economic importance the disease has been classified among the OIE list of diseases.

Infectious Bovine Rhinotracheitis is an acute febrile contagious viral disease caused by Bovine Herpes Virus-1. BHV-1 is a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae*, which belongs to the family *Herpesviridae*. It is an enveloped virus with double stranded DNA as genome. The capsid is icosahedral and is constructed of 162 capsomeres.

As other herpes viruses, BHV-1 is able to establish latent infections. Latency is characterized by the fact that no infectious virus can be isolated and no viral antigen can be demonstrated in latently infected cells. The latent virus represents a long term reservoir in an immune host, which becomes relevant on reactivation, virus can be excreted and transmitted to susceptible hosts. This poses problems in effective control of the disease.

Infection is easily transmitted because large quantities of the virus are shed in respiratory, ocular and reproductive secretions of infected cattle. The virus is perpetuated in bovine populations by direct contact between infected cattle and probably by latent infections that are occasionally reactivated, accompanied by virus shedding. Also bulls with balanoposthitis can transmit the disease during breeding. Virus from these lesions can contaminate the semen and constitute a hazard in natural breeding or artificial insemination. If semen is frozen, the virus is preserved.

The disease emerged as a major economic problem in dairy herds and beef feedlots in California and Colorado in 1950. First report was published in 1954 by Schroeder and Moys. Since then the disease has been reported in most countries of the world.

In India IBR was first reported by Mehrotra et al. (1976) who isolated the virus from cases of keratoconjunctivitis amongst cross bred calves in an organized cattle farm in Uttar Pradesh. Since then, widespread evidence of IBR has been reported from most states of the country. In Kerala, seropositive animals have been detected by Sulochana *et al*. (1982) and Rajesh *et al*. (2003).

Conventionally, the confirmed laboratory diagnosis of IBR involves the isolation of organism from affected tissues and secretions. Along with that, a battery of serological tests is applied to diagnose the disease. The serological tests used for detection of IBR viral antibodies include serum neutralization test (SNT), indirect Enzyme Linked Immuno Sorbent Assay, blocking ELISA, indirect haemagglutination (IHA), fluorescent antibody technique (FAT), complement fixation test (CFT) and agar gel immunodiffusion. Viral antigen can be detected using techniques like direct or indirect FAT, immunohistochemistry and ELISA. The advantages of antigen detection methods versus virus isolation are that cell culture facilities are not required and a laboratory diagnosis can be made in one day. Recently various polymerase chain reaction assays have been developed to detect BHV-1 DNA in clinical samples. The thymidine kinase, *gB*, *gC*, *gD*, and *gE* genes have been used as targets for amplification. Compared with virus isolation, the PCR has the primary advantages of being more sensitive and more rapid and it can be performed in 1-2 days. It can also detect DNA in latently infected sensory ganglia.

Keeping the above facts in view, the present study was undertaken with the following objectives:

1) To detect Infectious Bovine Rhinotracheitis (IBR) virus from apparently healthy and clinically ill bovines by immunofluorescence and polymerase chain reaction

2) Attempts to isolate the IBR virus

Review of literature

2. REVIEW OF LITERATURE

Infectious Bovine Rhinotracheitis (IBR) is a highly infectious disease of cattle that is caused by virus belonging to herpes group (Armstrong *et al*., 1961).

Bovine Herpes Virus-1 (BHV-1), also called Infectious Bovine Rhinotracheitis virus, produces a number of clinical manifestations in cattle, *viz*, respiratory diseases, keratoconjunctivitis, abortion and reproductive disorders (Kahrs, 1977).

2.1. HISTORICAL BACKGROUND

Schroeder and Moys (1954) reported an acute respiratory tract infection of dairy cattle, characterized by high fever and sudden cessation of lactation, which appeared in Los Angeles in 1953-1954. The etiology of the disease was undetermined, but investigations indicated that could be caused by a virus.

Miller (1955) described a respiratory disease that was first seen in Colorado feedlot cattle in the fall of 1950. It was known by the names"red nose" and "dust pneumonia" and later it had been called "necrotic rhinotracheitis".

Madin *et al*. (1956) first isolated the etiological agent of IBR.

The genital form of the disease was referred to as Infectious Pustular Vulvovaginitis by Kendrick *et al*. (1958).

The disease was first reported in India by Mehrotra *et al*. (1976) who isolated IBR virus from cases of conjunctivitis in cross bred calves.

2. 2. SEROPREVALENCE

2. 2. 1. Abroad

Rampton *et al*. (1976) detected IBRV antibodies in the sera of ten species of wild life in East Africa.

Cerqueira *et al*. (2000) conducted a serological survey for Bovine Herpesvirus 1 in cattle from different regions in the state of Bahia, Brazil and suggested that the virus was circulating actively in the herds.

Kampa *et al*. (2004) reported a moderate level of exposure of dairy herds to Bovine Viral Diarrhoea (BVD) virus and BHV-1 in Northern and Northeastern Thailand, with prevalences of 73 per cent and 67 per cent respectively.

Tolga Tan *et al*. (2006) conducted a study to assess the prevalence of BHV-1 and Bovine Leukemia Virus (BLV) in selected dairy cattle herds in Aydin Province, Turkey and the results indicated that the prevalence was 19.5 per cent and 0.3 per cent respectively.

A serological survey of Bovine herpesvirus-1 conducted in beef herds selected from central and southern regions of Turkey by serm neutralization test revealed that 78.26 per cent of herd from central Turkey and all of the herds from southern Turkey had seropositive animals (Duman *et al*., 2007).

Yan *et al*. (2008) demonstrated an overall nation wide seroprevalence of 35.8 per cent for BHV-1 in China using gG antibody indirect ELISA.

2. 2. 2. In other states in India

Aruna and Suribabu (1992) carried out an investigation to ascertain the extent of prevalence of IBR in buffaloe population in Andhra Pradesh, by employing indirect haemagglutination and observed an overall prevalence of about 21.05 per cent.

The seroprevalence study of IBR using AB- ELISA on 3428 bovines from 18 states of India and union territory of Andaman & Nicobar Islands revealed that 38.01 per cent of animals contained antibodies to BHV-1 (Suresh *et al*., 1999).

Chinchkar *et al*. (2002) studied the seroprevalence of IBR in Maharashtra state and found that about 32.26 per cent of samples were positive.

Dhand *et al*. (2002) undertook a study to find out the seroprevalence of IBR in Punjab using AB-ELISA and found that the prevalence was lower in unorganized farms and species wise prevalence was significantly higher in cattle compared to buffaloe.

Aradhana *et al*. (2004) designed a study to assess the status of IBR in Punjab and reported a seroprevalence of 31.8 per cent and also observed a statistically significant relationship between repeat breeding and IBR.

Nandi *et al*. (2004) reported Bovine Herpesvirus-1 seroprevalence of about 46.77 per cent in cattle and 62.96 per cent in buffaloes, after evaluating the serum samples of cattle and buffaloes in nine states along North and Central region of India by microserum neutralization test.

A serological survey of IBR conducted in cattle and buffaloe in Marathwada region of Maharashtra using indirect ELISA revealed a seroprevalence of about 8.9 per cent and emphasized the need of seromonitoring programme for IBR (Pharande *et al*., 2004).

An overall seroprevalence of 19 per cent had been reported in Mithun maintained at the National Research Center on Mithun, Nagaland (Rajkhowa *et al*., 2004).

Sunder *et al*. (2005) conducted a study to determine the incidence and prevalence of livestock diseases in Andaman & Nicobar Islands and the results revealed 13.83 per cent prevalence of IBR.

The overall seroprevalence of BHV-1 antibodies in breeding bulls in Gujarat was found to be 29.21 per cent by M-ELISA test (Jain, 2006).

The magnitude and pattern of occurrence of IBR in the livestock reared in the Garhwal region of Uttaranchal had been studied using AB-ELISA and established an overall prevalence of 10.39 per cent (Jain *et al*., 2006).

The seroprevalence of IBR among cattle in Himachal Pradesh was 50 per cent (Sharma *et al*., 2006)

Koppad *et al*. (2007) undertook a study to know the seroprevalence of IBR in Karnataka and the results revealed an overall prevalence of 19.2 per cent.

Rahman *et al*. (2007) reported that the overall seroprevalence of IBR in yaks in India was 40.80 per cent and the prevalence was higher among females than males.

2. 2. 3. In Kerala

Sulochana *et al*. (1982) conducted a serological survey on the occurrence of IBR in Kerala and found that 56.84 per cent of animals with reproductive disorders and 47.43 per cent of clinically normal animals were seropositive.

Rajesh *et al*. (2003) undertook a study to assess the seroprevalence of IBR among cattle population in Kerala using AB-ELISA and the results revealed a seroprevalence of about 14.88 per cent.

2.3. ETIOLOGY

2. 3. 1. Taxonomy

Based on morphological features and physicochemical properties, BHV-1 is a member of the family *Herpesviridae* (Armstrong *et al*., 1961)

Bovine Herpes Virus-1 belongs to the subfamily *Alphaherpesvirinae* (Gibbs and Rweyemamu, 1977).

Mckercher *et al*. (1959) conducted a study to compare the etiological agents of IBR and Infectious pustular vulvovaginitis (IPV) and confirmed that the viruses causing IBR and the one causing IPV are one and the same.

Osorio *et al*. (1985) compared the reference strains and field isolates of herpes viruses recovered from cattle by restriction enzyme analysis and indirect FAT and defined five major subtypes of BHV, *viz*, infectious bovine rhinotracheitis virus, bovine herpes mammilitis virus, malignant catarrhal fever virus, bovine cytomegalovirus candidate and the syncytia forming Pennsylvania-47 strain.

2. 3. 2. Properties of the virus

2. 3. 2.1. Morphology

2. 3. 2. 1a. Virion structure

Herpesvirus virions are enveloped, about 150nm in diameter and contain an icosahedral nucleocapsid about 100nm in diameter, composed of 162 hollow capsomeres. The DNA genome is wrapped around a fibrous spool like core, which has the shape of a torus. Surrounding the capsid is a layer of globular material, known as tegument, which is enclosed by a typical lipoprotein envelope with numerous small glycoprotein peplomeres (Murphy *et al*., 1999).

2. 3. 2.1b. Genome

The G+C content of the BHV-1 genome ranges from 71 – 72 per cent (Gibbs and Rweyemamu, 1977)

Seal *et al.* (1985) described that although the DNA of the BHV-1 isolates from different types of infections might vary in their restriction enzyme patterns, the liquid hybridization studies indicated that DNA sequences were at least 95 per cent homologous.

Mittal and Field (1989) determined the nucleotide sequence of *TK* gene of Bovine Herpesvirus-1 strain 6660.

Smith *et al*. (1990) performed restriction endonuclease digestion to find out the location of the thymidine kinase gene of bovine herpesvirus type 1.2 and also determined the nucleotide sequence of BHV 1.2a *TK* gene.

The BHV-1 genome sequence comprises 67 unique genes and two genes, both duplicated, in the inverted repeats. Thus BHV-1 encodes at least 69 proteins (Schwyzer and Ackermann, 1996).

2.3.2.1c.Viral proteins

An electrophoretic analysis of IBR virus revealed the presence of 25 to 33 structural polypeptides, of which a total of 11 were identified as glycoproteins (Misra *et al*., 1981).

Bolton *et al*. (1983) performed sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) to identify the proteins of infectious bovine rhinotracheitis virus and classified viral proteins as envelope proteins, envelope-associated proteins and nucleocapsid proteins.

Marshall *et al*. (1986) characterized the envelope glycoproteins of IBR virus by biochemical and immunological methods and the results suggested that the major BHV-1 glycoproteins involved in viral neutralization in vitro are the 150K/77K and 180K/97K and to a lesser extent the 130K/74K/55K protein.

The BHV-1 genome consists of at least 10 different genes with the potential to encode glycoproteins gB, gC, gD, gE, gI, gH, gL.gG, gK and gM. The predicted molecular weights of the corresponding gene products range from 17- 101 kDa. Their observed sizes were larger because N- as well as O- linked oligosaccharides were attached and they might form homo and hetero dimmers (Schwyzer and Ackermann, 1996).

Rijsewijk *et al*. (1999) found that the epitopes on glycoprotein C of BHV-1 could be used to differentiate between BHV1.1 and BHV 1.2 that cause IBR and IPV respectively.

2. 3. 2.2. Physicochemical properties

Crandell *et al*.(1975) determined the sensitivity of 12 field isolates of IBRV and four commercial modified live vaccine strains, after exposure to ether and differences were demonstrated in the ether sensitivity of both field and vaccine strains.

Elazhary and Derbyshire (1979) demonstrated that IBR virus was able to survive well enough in atmosphere for airborne transmission of the infection to occur and the most favorable conditions for short term survival of the virus seemed to be low temperature and high relative humidity. The more rapid decay of IBRV at acidic pH was also noted.

2. 3. 2. 3. Latency

Pastoret *et al*. (1980) attempted reactivation of temperature sensitive and non-temperature sensitive IBR vaccine virus from animals, by the use of dexamethazone and suggested that both type of vaccines could establish latency in animals.

Ackermann and Wyler (1984) demonstrated the presence of IPV virus DNA in local sensory ganglia during latency, by *in situ* hybridization.

Latency is characterized by the fact that no infectious virus could be isolated and no viral antigen can be demonstrated in latently infected cells (Engels and Ackermann, 1996).

Lemaire *et al*. (2000) demonstrated for the first time that BHV-1 seronegative latent carriers could be obtained by infecting young calves,protected by maternal immunity, with a virulent BHV-1 strain.

Tonsil is a site for the persistence or latency of BHV-1 from which the virus can be reactivated by dexamethazone and the shedding of virus from tonsil during reactivation plays a major role in virus transmission (Winkler *et al*., 2000).

Wang *et al*. (2001) detected BHV-1 in peripheral blood mononuclear cells, eight months post infection by PCR and *gD* immunofluorescence and concluded that the lymphoid tissues might also function as sites of latency.

2. 4. PATHOGENESIS

Forman *et al*. (1982) designed a work to determine the susceptibility of bovine alveolar macrophages to infection with IBRV and IBRV replication in bovine alveolar macrophages was demonstrated by a variety of methods.

Ohmann and Babiuk (1986) suggested that following aerosol infection with BHV-1, the percentage of alveolar macrophages expressing an MHC II antigen and

Fc mediated phagocytosis increased, as did the activity level of two ectoenzymes and the lysosomal hydrolase beta glucuronidase and the generation of PgE2 by the alveolar macrophages.

Meyer *et al*. (1998) described that BHV-1 gH was essential in the infectious cycle of the virus and was especially involved in virus entry and cell to cell spread.

Tanghe *et al*. (2005) suggested that the binding of BHV-1 to spermatozoa was mediated by the viral glycoproteins gC and gD, and therefore seemed to be comparable with the mechanisms of BHV-1 attachment to its natural host cell.

2. 5. EPIDEMIOLOGY

2. 5. 1. Host range

Karstad *et al*. (1974) isolated IBR virus from vaginal swabs of recently captured wildebeest and indicated that IBR virus could be carried by wildebeest in the absence of clinical signs.

Porter *et al*. (1975) isolated IBR virus from mink and ferret.

A virus, which was isolated from tissue samples from two still- borne pig fetuses, had been identified as a strain of IBRV on the basis of its morphology, cytopathology, physicochemical and serological characteristics (Derbyshire and Caplan, 1976).

Trueblood *et al*. (1978) isolated a virus from the trachea of a lamb that was suffering from a respiratory disorder and the physical and chemical properties of the isolate confirmed that it was a herpes virus.

An experiment conducted using neonatal striped skunks revealed that IBRV infection could be established in neonatal skunks. This finding extends the range of known susceptible hosts for IBR (Lupton *et al*., 1980).

Rock and Reed (1982) suggested that the pathogenesis of persistent BHV-1 infection in rabbits was similar to that thought to occur in cattle and this similarity indicated the usefulness of rabbits in the study of persistent BHV-1 infection.

Whetstone and Evermann (1988) characterized BHV-1 isolates obtained from sheep and goat and reported that sheep and goat should be considered potential hosts for BHV-1 and might be involved in the interspecies transmission of virus among domestic livestock.

Clark *et al*. (1993) isolated herpes viruses from nasal swabs taken from two peninsular bighorn sheep and restriction enzyme analysis pattern of these isolates was found to be similar to the Cooper strain of IBRV.

Varady *et al*. (1994) extracted DNA from BHV-1 isolated from a still borne porcine foetus and REA pattern was compared with bovine isolates of BHV-1. They concluded that the porcine virus was genetically similar to bovine isolates of BHV-1.

2. 5. 2. Factors affecting infection

2. 5. 2. 1. Age

The case fatality rate is considerably higher in susceptible neonates (calves below 2 weeks) than in adults (Kahrs, 1977).

2. 5. 2. 2. Immune status of the host

Mechor *et al*. (1987) reported that the feeding of colostrum from seropositive vaccinated cows protected the newborn calves against the fatal multisystemic form of IBR.

A study conducted to assess the virulence of BHV-1 isolate in hill bull calves with known immunological status revealed that the pathogenicity depended on their immune status (Mehrotra *et al*., 1987).

Bryan *et al*. (1994) diagnosed BHV-1 infection in calves that had received an intramuscular injection of modified live IBR-parainfluenza vaccine between birth and 3 days of age and suggested that exposure of neonates to infectious BHV-1 might pose a significant threat and risk of vaccine induced disease was accentuated if the dam's colostrums did not have adequate levels of neutralizing BHV-1 antibody.

2. 5. 2. 3. Stress

van Reenen *et al*. (2000) performed an experiment to study the impact of stress on responsiveness to infection with BHV-1 in veal calves and suggested that social isolation after previous group housing enhanced the level of stress- related neuroendocrine factors including cortisol, which in turn, diminished clinical signs and development of fever in socially isolated calves.

2. 6. DIAGNOSIS

2. 6. 1. Virus isolation

2. 6. 1. 1. Primary cell cultures

Madin *et al*. (1956) first isolated the etiological agent of IBR in tissue cultures of bovine embryonic kidney.

Gratzek *et al*. (1966) isolated a strain of IBR virus from Peyer's patches of a calf, which had died with lesions typical of mucosal disease.

Beck (1975) isolated IBR virus from brain and spinal cord of a cow with severe neurological lesions in bovine embryonic kidney cells.

Mehrotra *et al*. (1976) isolated IBR virus from cases of conjunctivitis in primary calf kidney cell monolayers.

2. 6. 1. 2. Continuous cell lines

Propagation of BHV-1 in Madin Darby Bovine Kidney cell line revealed that the entry of herpes virus into susceptible cells was by a process of fusion of virus and cell (Zee and Talens, 1971).

Singh *et al*. (1986) recovered two viral isolates, one each from aborted materials of a Jersey cow and semen of a Jersey bull, by employing Auburn University Bovine Embryonic Kidney (AU-BEK) and Bovine Turbinate (B Tuc) cell

lines and reported that these cell lines were more sensitive than Madin Darby Bovine Kidney (MDBK) cell line for isolation of IBR virus.

Madin Darby Bovine Kidney cell line was used to isolate IBR virus from nasal swabs and blood samples of cattle during two outbreaks of respiratory form of IBR (Singh *et al*., 1989).

Bovine herpes viruses were isolated from preputial swabs collected during the acute phase of balanoposthitis outbreak and also from semen collected before the appearance of clinical signs and emphasized the risk of using contaminated semen for artificial insemination (Weiblen *et al*., 1992).

Deka *et al.* (2005) isolated Bovine Herpesvirus-1 in MDBK cell line from the semen samples of breeding bull and observed a distinct CPE characteristic of herpes virus on second passage.

Wild *et al*. (2005) propagated BHV-1 in MDBK cell line and studies using fluorescent and electron microscopic techniques revealed that the capsids impaired the nuclear pores as a gateway to gain access to the cytoplsmic matrix. They also emphasized the ability of capsids to induce budding at any cell membrane, provided the fusion machinery was present and/or budding was not suppressed by viral proteins.

Zambre *et al*. (2005) isolated IBR virus from frozen semen samples in MDBK cell line after diluting the samples 1:10 in foetal bovine serum and the virus was identified by serum neutralization test.

There was a virus dependant induction of activity of telomerase, an enzyme uniquely specialized for telomeric DNA synthesis and regulates the proliferative capacity of mammalian cells, in MDBK cells productively infected by BHV-1, which was mediated by an IE gene (Pagnini *et al*., 2006).

Sontakke *et al*. (2008) isolated IBR virus in MDBK cell line from different clinical manifestations like, rhinitis, conjunctivitis, repeat breeding and abortion.

2. 6. 1. 3. Cytopathic effect (CPE)

Mehrotra *et al*. (1976) observed that the first sign of CPE appeared as rounding of individual cells, with shrinkage and an increase in cytoplasmic granules. At 48 hrs post inoculation characteristic 'bunch of grapes' like aggregation developed and at places the cell sheet detachment in clumps was prominent. Within 72-96 h cell destruction was complete and all the cell culture featured characteristic intranuclear inclusion of Cowdry's type A. These inclusions were separated by a clear zone or halo.

As with other alpha herpes viruses, there was a rapid cytopathic effect with syncytia and characteristic eosinophilic intranuclear inclusion bodies (Murphy *et al*. 1999).

2. 6. 2. Detection of viral antigen

2. 6. 2. 1. Enzyme Linked Immuno Sorbent Assay (ELISA)

An antigen capture ELISA was developed for the detection of BHV-1 in nasal swab specimens (Collins *et al*., 1985).

Collins *et al*. (1988) reported that a rapid antigen capture ELISA was useful for the diagnosis of BHV-1 respiratory infection.

2. 6. 2. 2. Fluorescent antibody test (FAT)

Peter *et al*. (1966) used fluorescent antibody test to detect BHV-1 antigen in tissues of calves experimentally inoculated with IBR Iowa State University strain-I.

Reed *et al.*, (1971) performed FAT to diagnose abortion caused by IBR virus under field conditions and found that fluorescence specific to IBR virus was always focal and most prevalent and brilliant in foetal kidney sections.

An experimental study was conducted to investigate the use and reliability of FAT on nasal epithelial cells for the diagnosis of IBR and BVD in cattle and the results suggested that the sensitivity of FAT was comparable to that of cell culture method and FAT could be directly applied on smears of swabs from nasal passages of infected animals (Silim and Elazhary, 1983).

Schipper and Chow (1968) reported that IBR could be readily and accurately diagnosed by direct FAT on infected bovine embryo kidney monolayers.

El-kholy (2005) detected BHV-1 antigens in infected MDBK cells using indirect immunofluorescence.

2. 6. 2. 3. Immunoperoxidase test

Miller and Maaten (1989) demonstrated IBR virus antigen in paraffin sections of tissues of aborted fetuses from experimentally infected heifers by

immunohistochemistry and suggested that this technique might be useful in diagnostic laboratories to detect IBR virus infection in tissues that were not suitable for virus isolation or examination by tissue section FAT.

An immunoperoxidase procedure was developed using specific monoclonal antibody and an avidin- biotin- peroxidase complex to detect IBR virus antigen in formalin fixed paraffin- embedded tissue sections (Smith *et al*., 1989).

Haines and Clark (1991) used immunohistochemistry to demonstrate BHV-1 in tissues of a calf died following vaccination with modified live virus vaccine against BHV-1.

Bulut *et al*. (1998) demonstrated IBR virus antigens in infected MDBK cells by immunoperoxidase staining.

2. 6. 3. Detection of antiviral antibodies

2. 6. 3. 1.Enzyme Linked Immuno Sorbent Assay

Enzyme Linked Immuno Sorbent Assay is a superior choice for the diagnosis of IBR virus infections because of its ability to detect non-neutralizing antibodies, its great sensitivity and low cost (Bolton *et al*., 1981)

Cho and Bohac (1985) developed an ELISA for the detection of IBR viral antibody in cattle and reported that ELISA was a specific, sensitive and practical test for the detection of anti IBR viral antibodies.

Florent and De Marneffe (1986) applied an ELISA to detect serum antibodies against IBR, parainfluenza-3, adeno virus type-3 and bovine respiratory syncytial virus.

A differential diagnostic blocking ELISA was developed for use in conjunction with modified-live IBRV gIII deleted marker vaccine and suggested that it was sensitive and specific and could be useful in distinguishing serologically between vaccinated cattle and those infected with IBRV field strains (Kit *et al*., 1993).

Kramps *et al*. (1994) developed a simple highly sensitive blocking ELISA for the detection of antibodies to BHV-1.

Enzyme Linked Immuno Sorbent Assay+s were standardized for the quantitative estimation of antibodies specific for IBRV, respiratory syncytial virus, PI-3 and BVD viruses to give quantitative result when testing was performed at single optimum dilution (Graham *et al*., 1997)

Pharande *et al*. (2004) used indirect ELISA to study the seroprevalence of IBR among cattle and buffaloe in Marathwada region of Maharashtra.

Sarumathi *et al*. (2004) compared serum and milk based Avidin- Biotin ELISA for the detection of antibodies to IBR and concluded that milk samples could be used as an alternative for sera in detecting antibodies to IBR virus and could be used in mass screening programmes.

Jain *et al*. (2006) studied the seroprevalence of IBR in the livestock reared in Uttaranchal using AB-ELISA.

2. 6. 3. 2. Fluorescent antibody test (FAT)

Assaf *et al*. (1975) reported that immunofluorescence on rabbit kidney cells was as sensitive as neutralization test for the detection of antibodies to IBR.

Graham *et al*. (1999) described an immuno fluorescent antibody test to detect BHV-1 specific IgM and opined that it was a useful technique for the serodiagnosis of BHV-1 infection.

2. 6. 3. 3. Virus/serum neutralization test

Serum neutralization test is more sensitive and specific and when used for diagnostic purpose, it would be advisable to use more replicates per serum and one of the standard methods of calculating 50 per cent SN end points to increase the accuracy of the test (Rossi and Kiesel, 1971).

Potgieter and Mare (1974) distinguished different strains of infectious bovine rhinotracheitis virus by neutralization kinetics with late 19s rabbit antibodies.

Potgieter (1975) carried out kinetic neutralization with 7s and 19s globulins derived from sera of calves collected after inoculation with IBRV, both in presence and absence of guinea pig complement (C) and found that the 19s neutralizing antibody was dependant on guinea pig complement. He also reported that neutralizing activity was readily detected in 19s globulins from early sera and not in any of late sera and concluded that it maight be possible to determine whether cattle have recently been exposed to IBRV by determining the presence of C dependant globulins.

Rampton and Jessett (1976) performed virus neutralization test to detect IBR virus antibodies in the sera of wild life and the immune serum was titrated using the variable serum-constant virus technique.

Deregt *et al*. (1993) evaluated two sensitive neutralization tests, which differed in the presence or absence of complement, for the detection of antibodies to BHV-1 in bovine sera and found that the test in presence of complement was more sensitive than that in absence of complement for detecting an early immune response after experimental infection.

Micro serum neutralization test was performed in monolayers of MDBK cells in micro plates, using Los Angeles BHV-1 strain, to assess the prevalence of IBR in cattle from different regions in the state of Bahia, Brazil (Cerqueira *et al*., 2000).

2. 6. 4. Molecular methods

2. 6. 4. 1. DNA extraction

van Engelenburg *et al*. (1995) lysed the seminal fluid at 60 ^{0}C for 60 min using sodium chloride, Tris, EDTA, Tween 20, proteinase K and bacteriophage lamda DNA. The DNA present in the lysate was directly purified by chromatography.

Non-idet P-40 (NP-40) and proteinase K were used for extraction of DNA of BHV-1 from semen samples (Masri *et al*., 1996).

Tiwari *et al*. (2000) performed PCR to detect BHV-1 from infected cell culture supernatant, extracting DNA simply by heating in boiling water bath without further extraction or purification of DNA and compared it with PCR on viral DNA purified

by SDS-proteinase K lysis followed by phenol: chloroform extraction and precipitation by ethanol and concluded that phenol:chloroform extraction method increased the sensitivity of PCR.

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. The spermatozoa free supernatant was then subjected to proteinase K treatment and phenol: chloroform extraction.

2. 6. 4. 2. Polymerase chain reaction

van Engelenburg *et al*. (1993) developed a rapid and sensitive PCR assay for the detection of BHV-1 in bovine semen and observed that this assay was more sensitive than the routinely used virus isolation method.

A PCR amplification method was designed using primers in thymidine kinase region for the detection of BHV-1 strains (Kibenge *et al*., 1994).

Vilcek *et al*. (1994) detected BHV-1 DNA using a PCR assay based on primers from the viral *gI* gene to generate a product of size 468 bp and observed that the sensitivity limit for detection by PCR is 3 fentogram of pure DNA, which represents approximately 20 viral genomes.

van Engelenburg *et al*. (1995) experimentally inoculated bulls with BHV-1 intrapreputially, to compare the sensitivities of PCR and virus isolation and to examine the course of virus excretion in semen. He found that PCR assay was more sensitive than virus isolation and the infected bulls excreted BHV-1 in semen much longer.

Gee *et al*. (1996) used PCR assay for the detection of BHV-1 in semen of bulls, which were seropositive for IBR and reported that the assay was more sensitive than virus isolation.

A nested PCR was developed for the detection of BHV-1 in bovine semen and comparison with virus isolation indicated that PCR assay was both sensitive and specific (Masri *et al*., 1996).

Ashbaugh *et al*. (1997) developed a sensitive method for simultaneously detecting and discriminating between BHV-1 and BHV-5 using nested PCR technique.

Moreira (1998) reported that the use of agarose blocks containing embedded DNA improves PCR amplification from templates naturally contaminated with polysaccharides.

A sensitive PCR assay specific for *gE* of BHV-1 was designed to detect the viral DNA from whole blood samples and this technique allowed discrimination between wild type virus infected and vaccinated animals (Fuchs *et al*., 1999).

Candido *et al*. (2000) developed a PCR assay allowing the efficient amplification of genomic fragments of BHV-1 and concluded that addition of co-solvents like dimethyl sulfoxide, glycerol and NP-40 was successful in increasing the specificity of selected fragments of the BHV-1 gene.

Moore *et al*. (2000) described a rapid, sensitive and specific PCR assay for the detection of BHV-1 DNA without the need for prior virus isolation and the assay was

based on the selected amplification of a portion of the viral thymidine kinase gene so as to get a product of size 298 bp and determined the sensitivity limit as 1 $TCID_{50}$ per 50 microliter.

A simple PCR method was for the detection of BHV-1 from infected MDBK cells indicating that this method would save time and costly chemicals (Tiwari *et al*., 2000).

De-Giuli *et al*. (2002) developed a multiplex PCR method coupled with restriction analysis of PCR products for the simultaneous detection of BHV-1, BHV-2 and BHV-4 infections.

Bovine Herpes Virus-1 infection in breeding bull semen was detected by virus isolation and PCR based on *gI* gene of BHV-1 and the results suggested that PCR was more sensitive compared to virus isolation (Deka *et al*., 2005).

El-kholy (2005) performed a nested PCR utilizing *gB* gene of BHV-1 as target for the detection of BHV-1 DNA in suspected clinical specimens and determined the sensitivity limit of PCR for the detection of BHV-1 DNA versus viral concentration as $\geq 10^{3.6}$ $TCID_{50}$ per milliliter.

Rola *et al*. (2005) designed a PCR technique based on the amplification of conserved regions of the gene encoding gD glycoprotein of BHV-1 which was more sensitive than virus isolation and the specificity of PCR products was confirmed by restriction enzyme analysis.

Polymerase chain reaction was found to be more sensitive than direct fluorescent antibody test in detecting BHV-1 from semen (Jain, 2006).

The important sources of PCR inhibitors are the materials and reagents that come into contact with samples during processing or DNA purification (Bessetti, 2007)

2. 6. 4. 3. DNA hybridization

Dorman *et al*.(1985) designed a molecular hybridization technique using biotinylated DNA probes to detect BHV-1 nucleic acid immobilized on nitrocellulose and also determined optimal methods for the immobilization of infected cells, cell extracts and clinical specimen preparations on nitrocellulose.

An *in situ* hybridization method was developed with a biotinylated probe for the detection of BHV-1 in aborted foetal tissue and comparison with conventional methods like virus isolation, FAT and immunoperoxidase techniques revealed that it was more sensitive and specific (Ayers *et al*., 1989).

Xia *et al.* (1995) used dot blot hybridization for the detection of BHV-1 in bovine semen and found that undiluted semen was best for this technique.

2. 7. CHARACTERIZATION OF THE VIRAL ISOLATE

Restriction endonuclease (RE) analysis

Osorio *et al.* (1985) compared reference strains and field isolates of herpes viruses recovered from cattle by restriction endonuclease analysis using the enzymes *Bam HI*, *Eco RI*, *Hind III* and *Bgl II*.

Whetstone and Evermann (1988) characterized bovine herpes viruses isolated from sheep and goat by RE analysis and radioimmunoprecipitation and confirmed that the isolate was IBRV and there was antigenic relationship between IBRV, IPVV and BHV-6.

Central nervous system isolates of BHV-1, which were recovered from bovine brain samples, were identified as BHV-1.3 by comparing the RE pattern with reference strain (d'Offay *et al.,* 1993).

Keuser *et al*. (2004) characterized two strains of caprine herpes virus type 1 (CpHV-1) by comparing the RE pattern with that of BHV-1 and CpHV-1 reference strains by employing the enzymes *Bam HI*, *Bst E II* and *Kpn I*.

Rola *et al.* (2005) used the restriction enzymes *BglI, AluI, AvaI, BbvI, DraIII* and *MnII* for confirming the specificity of PCR products.

Materials and Methods

3. MATERIALS AND METHODS

Molecular grade chemicals procured from M/s Genei, Bangalore and analytical grade chemicals purchased from M/s Sisco Research Laboratories Private Limited (SRL) and HiMedia Laboratories, Mumbai were used, wherever source is not mentioned. Glassware of Borosil brand and plastic ware of Tarson brand were used in this study.

3. 1. COLLECTION OF SAMPLES

A total of 60 samples, including 41 nasal swabs, 15 vaginal swabs, aborted materials from 5 cases and 4 semen samples were collected from suspected animals. Samples were collected mainly from the animals brought to the Veterinary hospital, Mannuthy, animals maintained in farms attached to Kerala Agricultural University and animals reared in certain private dairy farms in Thrissur. Majority of the animals had a history of respiratory infection and some had reproductive problems. Five samples were collected from apparently healthy animals. The swabs and aborted materials were immersed in Eagle's Minimum Essential Medium (MEM) containing 200 IU per ml of penicillin and 200µg per ml of streptomycin. The samples for virus isolation and PCR were stored at -20 ^{0}C pending use and that for immunofluorescence were processed within 6 hours.

Semen samples were collected in screw capped plastic vials from breeding bulls maintained in Artificial Insemination Centre, College of Veterinary & Animal Sciences, Mannuthy and transported on ice to the laboratory and subjected to immunofluorescence and molecular techniques. The semen samples were stored at -20 ^{0}C for future use.

3. 2. DETECTION OF VIRAL NUCLEIC ACID

3. 2. 1. Reference DNA

The IBR virus DNA procured from PD-ADMAS, Bangalore was used as reference for PCR.

3. 2. 2. Extraction of DNA

3. 2. 2. 1. Materials

3.2. 2. 1a. 10 per cent sodium dodecyl sulphate (SDS)

3.2. 2. 1b. Proteinase K

3.2. 2. 1c. Phenol:chloroform:isoamyl alcohol (25:24:1)

Phenol	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

3.2. 2. 1d. Chloroform:isoamyl alcohol(24:1)

Chloroform	24 ml
Isoamyl alcohol	1 ml

3.2. 2. 1e. Sodium acetate (3M, pH 5.5)

Sodium acetate	8.1648 g
Triple distilled water	20 ml

3.2. 2. 1f. Isopropanol

3.2. 2. 1g. 70 per cent ethanol

3. 2.2. 2. Method

3. 2. 2. 2a. Processing of clinical specimens

The swabs were thoroughly agitated and squeezed to recover maximum quantity of the sample. Then swabs were discarded and the medium was centrifuged at 1000 x g for 10 minutes. The tissue samples were homogenized with the addition of MEM to obtain 10 per cent suspension. The supernatant from swabs and specimen suspensions were used for viral DNA extraction. The semen samples were also diluted in MEM for DNA extraction.

The DNA was extracted from the samples as per the procedure described by Deka *et al*., (2005). About 500 µl each of the swabs and specimen suspensions as well as semen diluted in MEM were treated with 10 µl of 10 per cent SDS and 8 µl of proteinase K (250µg per ml). The mixture was incubated at 56 ^{0}C for one hour in a water bath, then mixed with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 15,000 x g for 15 minutes. The aqueous fraction was subjected to one cycle of chloroform: isoamyl alcohol treatment. Finally, after overnight incubation at -20 ^{0}C, the DNA was precipitated with 1/10 th volume of 3M sodium acetate and equal volume of isopropanol by centrifugation at 15,000 x g for 15 minutes. The DNA pellet was washed twice with 500 µl of 70 per cent ethanol (at -20 ^{0}C) by centrifugation at 15,000 x g for 15 minutes, then air dried and resuspended in 50 µl of ultra pure water for use in PCR.

3. 2. 3. Polymerase chain reaction

3. 2. 3. 1. Materials

3.2. 3. 1a.Taq DNA polymerase (3units per µl)

3.2. 3. 1b. Magnesium chloride (25mM)

3.2. 3. 1c. dNTP mix (2.5mM each)

3.2. 3. 1c. Extracted DNA

3.2. 3. 1d. 10 X PCR buffer

3.2. 3. 1e. Primers

Two oligonucleotide primers corresponding to nucleotide numbers 404- 424 and 701-681 respectively on BHV-1.1 thymidine kinase gene were used (Moore *et al*., 2000). The desalted primers were synthesized and supplied by M/s Genei, Bangalore.

Forward primer

5/- TGGTACGGACGCCTTAAGTGG -3/

Reverse primer

5/- GTTGATCTCGCGGAGGCAGTA -3/

3.2.3.1f. Triple distilled water

3.2.3.1g. DNA molecular weight marker

100 bp DNA ladder (M/s Genei,Bangalore) containing double stranded DNA segments of 100, 200,300,400, 500 and 600 bp.

3.2.3.1h. Reference DNA

3.2.3.2. Method

3.2.3.2a. Standardization of the protocol for PCR

The PCR reaction was carried out as per the method of Moore *et al*. (2000) with some modifications.

3.2.3.2b. Reconstitution of primers

The tubes containing lyophilized primers were centrifuged at 10000 x g for five minutes in a refrigerated centrifuge. To the tube containing forward primer 238 µl of triple distilled water was added and mixed well. From this 20 µl was taken and 80µl of triple distilled water was added. The reverse primer was also reconstituted in same manner. This reconstituted 20 picomole solution was used in PCR.

3.2.3.2c. Polymerase chain reaction

For standardization, the reference DNA was used in the reaction mixture as template DNA. The DNA was first heated to 100 ^{0}C for 5 minutes and then placed on

ice, followed by the addition of the PCR cocktail, which had been pre-heated to 90 ^{0}C for 2 minutes.

The reaction mixture (25µl) was prepared as follows:

10X PCR buffer	2.5 µl
Forward primer	1µl
Reverse primer	1µl
dNTP mix	2µl
$MgCl_2$	2.5µl
Taq DNA polymerase	0.4µl
Triple distilled water	10.6µl
Total	20µl
Template DNA	5µl

The amplification conditions were 95 ^{0}C for 5 minutes followed by 34 cycles of one minute at 95 ^{0}C, 50 s at 60 ^{0}C, 50s at 72 ^{0}C with a final extension step of 72 ^{0}C for 5 minutes.

Programme of amplification

One cycle	Denaturation	95 ^{0}C	5 min
34 cycles	Denaturation	95 ^{0}C	1 min
	Annealing	60 ^{0}C	50 s
	Extension	72 ^{0}C	50 s
One cycle	Extension	72 ^{0}C	5 min

3.2.4. Submarine agarose gel electrophoresis

3.2.4.1. Materials

3.2.4.1a. Tris – Acetate EDTA (TAE) buffer (50X), pH-8.0

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5M EDTA pH 8.0	20 ml
Distilled water upto	1000 ml

Autoclaved at 121^0C and 15 lb pressure for 15 minutes and stored at room temperature.

3.2.4.1b. Agarose gel (2 per cent)

Agarose low EEO (Genei)	2g
TAE buffer	100 ml

3.2.4.1c. Gel loading buffer

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Sucrose	40 g
Distilled water	100 ml

3.2.4.1d. Ethidium bromide

Ethidium bromide 100 mg
Distilled water 10 ml
Stored at 4 ^{0}C in amber coloured bottles.

3.2.4.2. Method

The PCR product was detected by electrophoresis in two per cent agarose gel in TAE buffer (1X). Agarose was dissolved in TAE buffer by heating. When the mixture cooled to 50 ^{0}C, ethidium bromide was added to a final concentration of 0.5 µg per ml (5µl per 100 ml). The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in proper position and the agarose was poured. Once the gel was set, the comb and adhesive tape were removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1X) was poured until the gel was completely covered.

The PCR product (5µl) was mixed with one micro liter of 6X gel loading buffer and the samples were loaded in the wells. Electrophoresis was carried out at 5V/cm until the dye migrated the full length of the gel.

The gel was visualized under ultraviolet transilluminator (Hoefer, USA) and the results were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.2.5. Processing of DNA extracted from samples

The DNA extracted from samples was subjected to PCR as per the method described earlier.

3.3 DETECTION OF VIRAL ANTIGEN

3.3.1. Indirect fluorescent antibody (IFA) technique

3.3.1.1. Materials

3.3.1.1a.Samples processed for the antigen detection

The swabs were processed within six hours of collection as follows. The epithelial cells were dispersed in the medium by shaking in a vortex shaker. After centrifugation of the suspension at 200 x g for eight minutes, the supernatant was decanted and the cells were washed in 5 ml of PBS pH 7.2 and centrifuged twice. The cells were resuspended in PBS and clumps of cells were removed by a pipette prior to placing 25 µl of suspension into a clean glass slide. The slides were air dried at room temperature and fixed in acetone for five minutes. They were rinsed briefly in deionized water to remove excess salts and dried again. The prepared specimens were stained immediately or stored at -20 ^{0}C until used.

The tissue specimens were processed as for DNA extraction, the supernatant was decanted and the cells were processed as described above.

Thin smears were directly prepared from semen samples and processed as described earlier.

3.3.1.1b. Calf anti- BHV-1 polyclonal serum (PD-ADMAS, Bangalore)

3.3.1.1c. FITC labeled anti-bovine IgG (VMRD, USA)

3.3.1.1d. Phosphate buffered saline (PBS, pH 7.6, 0.15M)

Sodium chloride	8 g
Potassium chloride	0.2 g
Potassium dihydrogen phosphate	0.2 g
Disodium hydrogen orthophosphate	1.15 g
Triple distilled water	1000 ml

Sterilized by autoclaving at 15 lb pressure at 121 ^{0}C for 15 minutes.

3.3.1.1e. Glycerol (4:1 in PBS)

3.3.1.2. Method

The IFA technique was carried out according to El-Kholy (2005) with some modifications. The slides were treated with the reference calf anti-BHV-1 polyclonal antiserum (diluted 1:100 in PBS, pH 7.6) for one hour. Then they were washed with PBS and probed with fluorescein isothiocyanate labeled antibovine IgG (diluted 1:50 in PBS). Again washed in three changes of PBS, mounted with glycerol (4:1 in PBS) and examined microscopically using a fluorescence microscope. A sample was considered positive when atleast two virus specific fluorescent cells were observed.

3.4. ISOLATION OF BHV-1 VIRUS

Madin Darby Bovine Kidney (MDBK) cell line obtained from National Centre for Cell Sciences, (NCCS), Pune, was employed for this purpose.

3.4.1. Subculturing and maintenance of cell line

3.4.1.1. Materials

3.4.1.1a. Sodium bicarbonate solution (7.5 per cent)

Prepared in triple distilled water and sterilized by filtration using 0.2µm membrane filter (Millipore, USA)).

3.4.1.1b. Calcium Magnesium free PBS (CMF-PBS, 0.15M, pH 7.2)

Dehydrated CMF-PBS was reconstituted as per manufacturer's instruction and sterilized by autoclaving at 15 lb pressure and 121 ^{0}C for 15 minutes.

3.4.1.1c. Trypsin –Versene Glucose (TVG) solution (working solution)

Trypsin (1:250)	0.25 g
EDTA	0.25 g
Glucose	0.05
CMF-PBS (0.15M, pH 7.2)	100 ml

Sterilized by filtration through 0.2 µm membrane filter, distributed in small aliquots and stored at -20 ^{0}C and prewarmed before use.

3.4.1.1d. Fetal calf serum

3.4.1.1e. Antibiotics

Benzyl penicillin	10 lac units

Streptomycin sulphate 1g
Sterile triple distilled water 100 ml

Distributed in aliquots and stored at -20 ^{0}C. It was used both in growth and maintenance medium at a level of 1 ml for every 100 ml of medium with a final concentration of 100 IU of penicillin and 100 μg of streptomycin sulphate per milliliter of the medium.

3.4.1.1f. Eagle's Minimum Essential Medium (MEM)

Dehydrated MEM was reconstituted as per manufacturer's instructions. Antibiotic solution was added and the medium was stirred well. Sodium bicarbonate solution was added and the pH was adjusted to 7.2. The medium was sterilized in positive pressure filtration assembly with 0.2 μm membrane filter. The medium was checked for sterility and stored at 4 ^{0}C until further use.

3.4.1.1g. Growth medium

The growth medium was prepared just at the time of use.
MEM with Earle's salts 90 ml
Fetal calf serum 10 ml

3.4.1.1h. Maintenance medium

The maintenance medium was prepared in the same manner as that of growth medium except that fetal calf serum was added at two per cent level.

3.4.1.1i. Tissue culture bottle of 50 ml capacity

3.4.1.2 Method

The maintenance medium was poured off from tissue culture bottle containing confluent monolayer. The cell sheet was washed with CMF-PBS. Two milliliters of prewarmed TVG solution was added to the bottle containing monolayer and then shaken gently for one minute. Then the TVG solution was discarded and the bottle was incubated at 37^0C for five minutes. When the cells started dislodging from the monolayer, added a small quantity of freshly prepared growth medium and the cells were detached from the surface by mechanical disruption using sterile pipette attached with bulb. A split ratio of 1:3 was employed for seeding into new tissue culture bottles and the cells were also seeded into test tubes containing coverslips. The tissue culture bottles and the test tubes containing coverslips were enriched with growth medium at the rate of eight milliliters and two milliliters respectively. The bottles were incubated at 37^0C. They were observed daily for the formation of monolayer. When monolayer was formed (usually within three to four days), it was used for further infection with BHV-1.

3.4.2 Inoculation of cell lines

3.4.2.1. Materials

3.4.2.1a. Fresh monolayer of MDBK cells in tissue culture bottles and cover slips

3.4.2.1b. Samples processed for inoculation

The sample that was positive for IFA was used for virus isolation. Specimen was processed as for DNA extraction and the supernatant was used for inoculation after filtering through 0.2 μm membrane filter.

3.4.2.2. Method

Tissue culture bottle with fresh monolayer was selected. The growth medium was poured off and washed with CMF-PBS. To this, one ml of inoculum was added and and incubated at 37^0C for one hour, to facilitate adsorption. Following this adsorption period, the inoculum was poured off, washed again with CMF-PBS, replaced with eight milliliters of maintenance medium. Control culture bottles were prepared simultaneously in which CMF-PBS was used as inoculum instead of clinical samples.

All the tissue culture bottles were incubated at 37^0C and were examined at 24 h interval for a period of seven days, under an inverted microscope for evidence of any cytopathic effect (CPE).

For infecting cover slip cultures in the test tubes 0.2 ml of inoculum and two ml of maintenance medium were used. Control tubes were also treated in the same manner in which CMF-PBS was used as inoculum. Infected cover slip cultures were collected at 24 h interval for a period of seven days, for studying the CPE by May-Grunwald Giemsa staining. The control tubes were also stained and studied.

3.4.3. Passaging of virus

3.4.3.1. Materials

3.4.3.1a. Inoculated monolayer in tissue culture bottles

3.4.3.1b. Fresh monolayer of MDBK cells in tissue culture bottles and cover slips

3.4.3.2.Method

Seven days post inoculation, the inoculated monolayer was freeze thawed and centrifuged at 2000 x g for 15 minutes in a cooling centrifuge (Remi C-24) to sediment cell debris. The supernatant was used as inoculum for next passage. The inoculation was performed as mentioned earlier (3.4.2.2). After every passage, the supernatant was tested for the presence of BHV-1 by PCR. Immunofluorescence was also performed to detect BHV-1 antigen in cells.

3.4.4 Staining of cover slip cultures

3.4.4.1. Materials

3.4.4.1a. Cover slip cultures

3.4.4.1b. May-Grunwald stain

Prepared by dissolving 2.5 g of May-Grunwald stain powder in 100 ml of absolute methanol and allowed to age for one month.

3.4.4.1c.Giemsa stain

Prepared by dissolving one gram of stain powder in 66 ml of glycerol and kept at 60^0C till the stain powder got dissolved completely. Then added 66 ml of absolute methanol and kept for a day.

3.4.4.1d. Methanol

3.4.4.1e. Acetone

3.4.4.1f. Xylene

3.4.4.1g. DPX mountant

3.4.4.2. Method

The cover slip cultures were fixed overnight in methanol. They were stained for 10 minutes in May-Grunwald stain and for 20 minutes in 1 in 10 diluted Giemsa stain. The cover slips were rinsed rapidly in two changes of acetone and then in two parts of acetone and one part of xylene for one minute, cleared in two changes of xylene, two minutes each, dried and mounted with DPX on a clean grease-free glass slide and examined for CPE under microscope. The uninfected cover slips were also fixed as described above and studied in detail.

Results

4. RESULTS

In the present study, 65 samples were screened for the presence of IBR virus. The samples included 41 nasal swabs, 15 vaginal swabs, aborted materials from 5 cases and 4 semen samples. Majority of the animals had a history of respiratory infection and some had reproductive problems. Five samples were collected from apparently healthy animals. Samples were collected mainly from the animals brought to the Veterinary hospital, Mannuthy, animals maintained in farms attached to Kerala Agricultural University and animals reared in certain private dairy farms in Thrissur.

All the samples were subjected to PCR and indirect immunofluorescence to detect the presence of IBR virus. Attempts were made to isolate IBR virus in MDBK cell line from the sample tested positive by IFA.

4.1. STANDARDIZATION OF POLYMERASE CHAIN REACTION

It has been established that there is high degree of sequence homology among *TK* genes of IBR virus strains belonging to BHV-1.1 and BHV-1.2. Hence *TK* (Thymidine Kinase) gene based primers that would be capable of detecting most of the BHV-1 strains, were used in this study. Thymidine kinase has been shown to play a role in viral pathogenicity, and therefore, DNA sequence encoding this enzyme is likely to be present in all pathogenic strains. Also, part of *TK* gene is deleted in BHV-5, thus offering the potential to discriminate between this virus and BHV-1. The primers were selected to generate an amplicon of size 298bp. The optimization experiments established that a clear band was obtained when an annealing temperature of 60^0C, 2.5mM $MgCl_2$ concentration and 35 cycles were used. Hence, the same conditions were used in all the experiments. The double stranded DNA extracted from the samples was diluted to weaken the inhibitory substances present and subjected to PCR. The positive control produced amplicon of approximately

298bp size, which was observed by analyzing the electrophoresed gel under UV transillumination. The negative control did not reveal any amplification.

4. 2. DETECTION OF BHV-1 IN CLINICAL SAMPLES BY PCR

Among 65 samples tested, no sample was found positive. Twenty samples each from ULF, Mannuthy and CBF, Thumburmuzhi were screened for the presence of IBR virus and all the samples were found negative. The ten samples from Veterinary hospital, Mannuthy and eleven samples from a private farm at Pattikkad, were also found negative. (Fig. 1)

4. 3. STANDARDISATION OF INDIRECT IMMUNOFLUORESCENCE (IFA)

Smears were prepared from nasal and vaginal swabs, aborted foetal tissues and semen and subjected to indirect immunofluorescence using FITC conjugated polyclonal antibovine IgG to detect BHV-1 antigen. The specimens for immunofluorescence were processed within six hours of collection. Minimum background fluorescence was observed when the FITC conjugated polyclonal antibovine IgG was diluted 1:30 in PBS. Hence, this dilution was used for all the samples. Bright, granular and virus specific yellowish green fluorescence associated with cytoplasm was observed in positive case. Some epithelial cells showed diffused non-specific fluorescence. The sample was considered positive when atleast two virus specific fluorescent cells were observed in a field.

4. 4. DETECTION OF BHV-1 ANTIGEN IN CLINICAL SAMPLES BY IFA

Among the 65 samples tested, one sample was found positive. Twenty samples from ULF, Mannuthy were screened for the presence of BHV-1 antigen and one

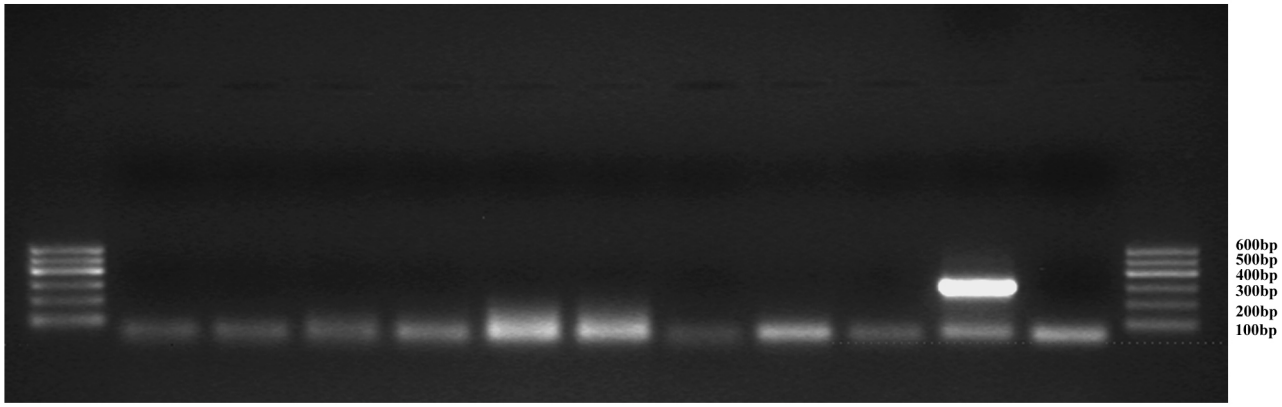

Fig 1. Agarose gel electrophoresis of PCR amplified product of IBR virus positive control.

Lane 1 & 13 - 100bp DNA ladder

Lane 2 to 10 - Samples

Lane 11 - Positive control

Lane 12 - Negative control

vaginal swab sample was positive (Fig.2 and Fig.3). Out of 20 samples from CBF, Thumburmuzhi, no sample was found positive. Among 10 samples screened from Veterinary hospital, Mannuthy and 11 samples tested from a private farm at Pattikkad all were found negative. No specific fluorescence was observed in negative control (Fig. 4).

4. 5. COMPARISON OF PCR AND IFA

Among the 65 samples tested, indirect immunofluorescence detected viral antigen in one sample. But PCR could not detect viral DNA in any of the samples.

4. 6. STANDARDIZATION OF CELL CULTURE TECHNIQUE

Madin Darby Bovine Kidney cell line procured from NCCS, Pune was used for virus isolation. Eagle's MEM containing Earle's salts, L- glutamine, sodium pyruvate and foetal calf serum was used as medium for the propagation of cell line. The cell line was incubated at 37^0C and 5 per cent CO_2 tension and examined under inverted microscope at 24 h interval. A monolayer was obtained within 72 h (Fig. 5). The monolayer was subcultured and fresh one was inoculated with the filtered sample.

4. 7. ISOLATION OF BHV-1 IN MDBK CELL LINE

Attempts were made to isolate BHV-1 in MDBK cell line, from the sample tested positive by immunofluorescence. No evidence of CPE could be appreciated in the sample even after third passage. After third passage attempts were made to extract DNA from the infected cell line and were subjected to PCR. But no specific amplification was obtained by PCR in this sample.

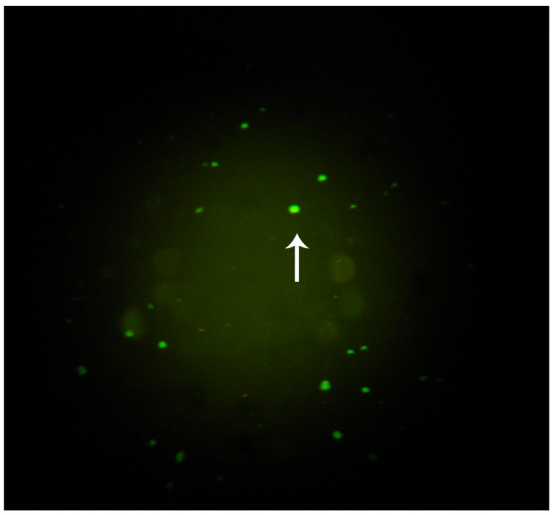

Fig 2. Vaginal epithelial cells infected with IBR virus after staining with FITC conjugated anti-bovine IgG showing yellowish green fluorescence (100X)

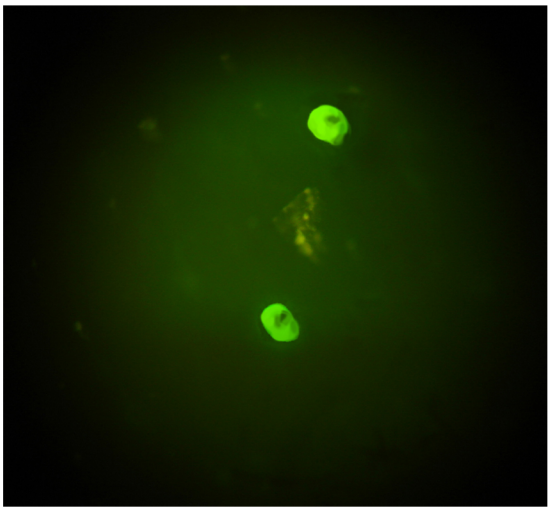

Fig 3. Vaginal epithelial cells infected with IBR virus after staining with FITC conjugated anti-bovine IgG showing yellowish green fluorescence (400X)

Fig 4. Fluorescent staining - Negative control

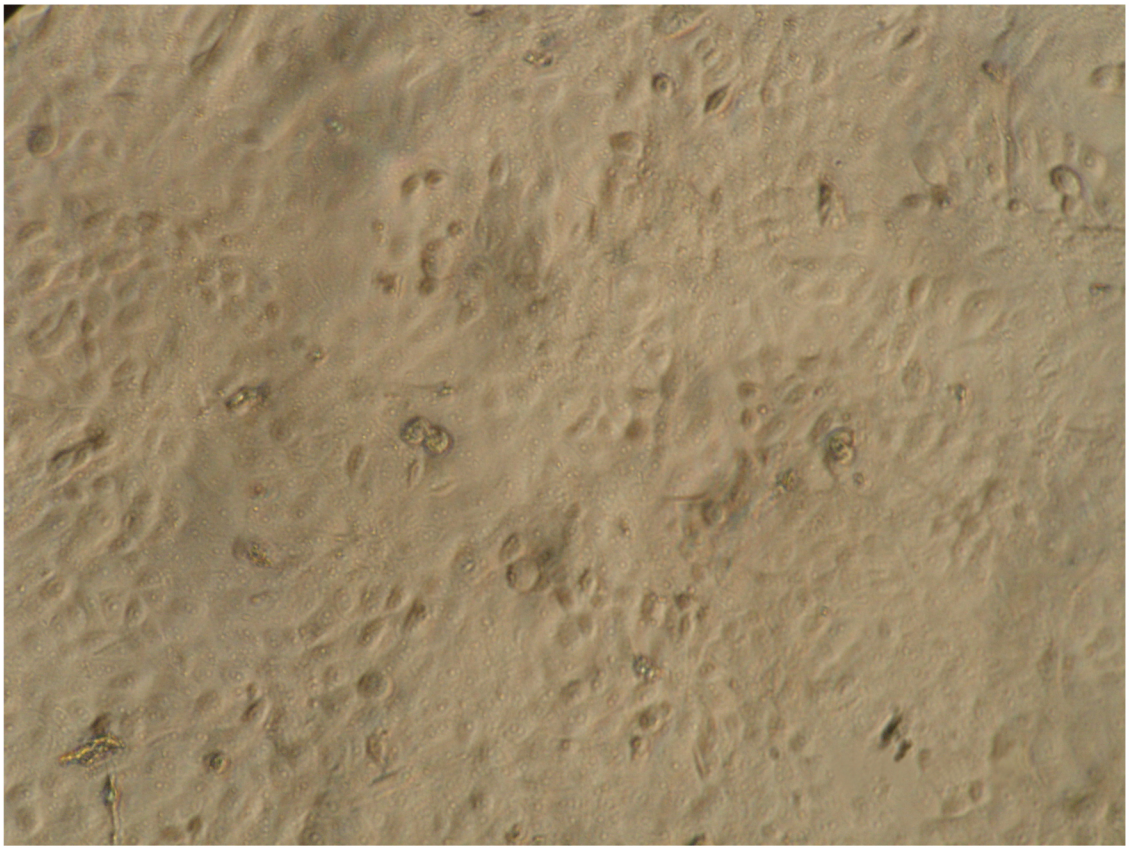

Fig 5. MDBK cell line (control)

Discussion

5. DISCUSSION

Infectious Bovine Rhinotracheitis emerged as a major economic problem in dairy herds in beef feedlots in California and Colorado, USA in 1954 (Schroeder and Moys). Shortly afterwards the causal virus was isolated and subsequently identified as a herpes virus (Madin, *et al*., 1956, Armstrong *et al*., 1961). The disease was first reported in India in 1976 (Mehrotra *et al*.). The virus causes several clinical manifestations including respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal infections (Gibbs and Rweyemamu, 1977).

Infection with BHV-1 is widespread, mainly because the virus remains in the host in a latent state after recovery from primary infection. It is thus difficult to establish the occurrence of recent infection in suspected clinical samples. Previously the diagnosis of IBR was mainly based on the isolation of the virus in tissue culture. Laboratory confirmation can also be obtained by serologic tests or fluorescent antibody staining to detect viral antigen. But paired serum samples are needed for serologic diagnosis and fluorescent antibody techniques are not always successful. Hence, these conventional methods are now being replaced by modern molecular techniques, which allow rapid and presumptive diagnosis. Several polymerase chain reaction assays have been developed to detect the presence of BHV-1, which are sensitive, specific and rapid. But false negative results cannot be excluded in case of PCR (Moore *et al*., 2000).

In the present study, PCR and immunofluorescence to detect BHV-1 in clinical samples were compared. The sample found positive by IFA was inoculated into MDBK cell line. But the attempts to isolate BHV-1 in cell line were unsuccessful.

5. 1. POLYMERASE CHAIN REACTION (PCR)

The PCR has become an important diagnostic tool for veterinary biologists. In addition to the speed, sensitivity and specificity, PCR offers an additional advantage in the case of BHV-1 that the present absolute requirement of good quality diagnostic samples is eliminated, given the fact that only short sequences of target nucleic acid are necessary for detection. Polymerase chain reaction was employed by many workers for the detection of BHV-1 (van Engelenburg *et al*., 1993, Vilcek *et al*., 1994, Fuchs *et al*., 1999 and Moore *et al*., 2000) using primers selected from highly conserved sequences encoding genes like *gB, gC, gD, gI* and *TK*. In the present study *TK* based primers were used to amplify the DNA extacted from clinical samples. No amplicon was generated in any of the samples. This could be due to the presence of inhibitory factors in clinical samples. According to Bessetti (2007), some inhibitors are inherent to the sample and other important sources of inhibitors are the materials and reagents that come into contact with samples during processing or DNA purification. Purification of DNA is the method used most often to remove inhibitors. Extraction methods that are proven to eliminate inhibitors from the purified DNA, such as, commercially available kits should be favoured. The choice of DNA polymerase can have a large impact on resistance to inhibition. Use of multiplex real time PCR to quantitate DNA provides an opportunity to use an internal positive control (IPC) to detect PCR inhibitors. Moreira (1998) demonstrated that the use of agarose blocks containing embedded DNA improves PCR amplification from templates naturally contaminated with polysaccharides, which is a powerful PCR inhibitor.

5. 2. INDIRECT IMMUNOFLUORESCENCE (IFA)

Immunofluorescence technique is commonly used in many laboratories to demonstrate viral antigens in tissue sections or inoculated cell cultures. Immunofluorescence on infected cell cultures sometimes requires two passages of the virus in cell cultures. This is costly and time consuming procedure. Silim and Elazhary (1983) used FAT directly on smears of swabs from nasal passages of infected animals and found that this method was slightly more sensitive than cell culture method. In the present study, indirect immunofluorescence was used on smears prepared from nasal and conjunctival swabs, aborted fetal tissues and semen samples using reference polyclonal antibovine IgG (FITC). The test detected viral antigen in one of the total 65 samples.

5. 3. COMPARISON OF PCR AND IFA

In the present study, out of 65 samples, all were tested negative by PCR. But IFA detected viral antigen in one sample. Jain (2000) also obtained similar result in case of bovine semen. Five semen samples, which were found positive by direct immunofluorecence, turned out to be negative by *gC*-PCR. This could be due to the presence of inhibitory factors in clinical samples. Silim and Elazhary (1983) reported that FAT was slightly more sensitive than cell culture method. According to the authors, because the specimens were processed same day, the epithelial cells were intact at the time of preparation of slides. This probably enhanced the sensitivity of immunofluorescence. In this study, washed suspension of cells was used for preparation of smear. This has the advantage of eliminating cell debris as well as mucus, thus reducing background fluorescence and intensifying the virus specific fluorescence. Moore *et al*. (2000) found that three samples, which were repeatedly negative by PCR, were scored positive by immunofluorescence. The authors

considered these as false negative PCR results and concluded that the samples may have contained only extensively degraded DNA or insufficient quantities of viral DNA for detection. In the present study also, the sample found positive by IFA did not reveal any amplification in PCR. This could be due to the presence of inhibitory factors in clinical sample, or the presence of insufficient quantities of DNA.

5. 4. ISOLATION OF BHV-1 IN MDBK CELL LINE

In the present study, attempts were made to isolate BHV-1 in MDBK cell line from the sample found positive by immunofluorescence. The sample was filtered before inoculation to avoid bacterial contamination of cell cultures. Singh *et al.* (1989) and Deka *et al*. (2005) successfully isolated BHV-1 in MDBK cell line from clinical samples.

Three passages were done with the sample. But no CPE could be observed. This is in accordance with the findings of Gee *et al*. (1996). According to them the failure of virus isolation in cell culture could be due to the presence of non-infectious virus in the samples. El-Kholy (2005) also got same results. Deka *et al*. (2005) observed a distinct CPE characteristic of herpesvirus on the second passage in MDBK cell line and Zambre *et al*. (2005) in the first passage. Moore *et al*. (2000) reported that some samples might contain viral inhibitors that interfere with viral isolation procedure.

In the present study, attempts were also made to isolate DNA from the cell culture and subjected to PCR. But no amplification could be detected in all the three passages indicating the absence of virus multiplication in the inoculated cell cultures.

Screening of more number of samples using PCR with primers based on different genes of BHV-1 and methods to overcome inhibitors will help in establishing a highly sensitive test for routine diagnosis of IBR. Given the wide range of PCR inhibitor-laden sample types and options available for handling them, a multifaceted approach may be the best solution for amplification failure.

Summary

6. SUMMARY

Infectious Bovine Rhinotracheitis, caused by Bovine herpesvirus-1, is world wide in distribution and manifest in a wide variety of clinical forms including conjunctivitis, infectious pustular vulvovaginitis, balanoposthitis, abortion and rarely encephalitis. In India the disease was first reported in 1976. Since then, seroprevalence has been reported from most of the states including Kerala. The disease causes severe economic losses, affecting the feed efficiency, milk production and reproduction of the infected animals. Hence, a successful control programme must be implemented, for which a reliable and rapid diagnostic test is essential. Conventional methods for diagnosis like virus isolation and immunofluorescence are less sensitive and time consuming. With the introduction of new molecular techniques like PCR, rapid and presumptive diagnosis has become possible. The present study was undertaken to detect the presence of IBR virus in clinical samples by PCR and immunofluorescence and to compare these techniques. Attempts were also made to isolate the virus in MDBK cell line.

A total of 65 clinical samples, including 5 from healthy animals were screened for the presence of IBR virus. Samples were collected from animals brought to Veterinary Hospital, Mannuthy, animals maintained in University Livestock farms, and also animals reared by some private dairy farmers in Thrissur district. All these samples were subjected to PCR and IFA.

The DNA was isolated from the clinical samples by phenol-chloroform-isoamyl alcohol method. The *TK* gene based primers were used to generate amplicon of size 298 bp. Among the 65 samples tested none was found positive by PCR. Smears were prepared from nasal and vaginal swabs, aborted foetal tissues and semen and subjected to indirect immunofluorescence using FITC conjugated polyclonal antibovine IgG. Immunofluorescence detected BHV-1 antigen in one of the samples.

Bright, yellowish green fluorescence was associated with cytoplasm in positive case. Polymerase chain reaction could not detect viral DNA in the sample found positive by immunofluorescence. This indicates that false negative results may occur in case of PCR and immunofluorescence on smears prepared from clinical samples can be considered as a rapid diagnostic method for IBR. Attempts were made to isolate BHV-1 from clinical samples in MDBK cell line. But no cytopathic effect could be detected even after third passage.

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*Originals not seen

Abstract

DETECTION OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS BY IMMUNOFLUORESCENCE AND POLYMERASE CHAIN REACTION

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ABSTRACT

A study was undertaken to detect the presence of IBR virus in clinical samples by PCR and immunofluorescence and to compare the efficacy of these tests. Attempts were also made to isolate the virus from clinical samples in MDBK cell line.

A total of 60 samples from suspected animals and 5 samples from healthy animals were collected from various sources like, University Livestock Farms, Veterinary Hospital, Mannuthy and some private dairy farms in Thrissur district. The samples included nasal and vaginal swabs, aborted materials and semen. All these samples were screened for the presence of IBR virus by PCR and immunofluorescence. The *TK* gene based primers were used for PCR and the positive control generated an amplicon of size 298bp. No sample was found positive by PCR. Immunofluorescence was performed on smears prepared from clinical samples using FITC conjugated polyclonal antibovine IgG. This test detected viral antigen in one of the samples, indicated by focal, bright yellowish green fluorescence associated with the cytoplasm of epithelial cells. The inability of PCR for detection of viral DNA in sample tested positive by immunofluorescence may be due to the presence of non-specific inhibitors in clinical sample. These results suggest that even though PCR is highly sensitive compared to other diagnostic tests, false negative results cannot be excluded. Immunofluorescence on smears prepared from clinical samples can be considered as a rapid diagnostic method for IBR. Attempts to isolate the virus from clinical samples in MDBK cell line were found unsuccessful.