

MOLECULAR STUDIES ON VIRUS
IDENTIFICATION AND SEROSURVEILLANCE
OF BOVINE HERPESVIRUS TYPE-1

By

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CERTIFICATE - I

This is to certify that the thesis entitled "Molecular Studies on Virus Identification and Serosurveillance of Bovine Herpesvirus type - 1 " submitted for the degree of Master of Veterinary Science, in the subject of Veterinary Microbiology to the Haryana Agricultural University, Hisar, is a bonafide research work carried out by Namrata Pandita under my supervision and that no part of this thesis has been submitted for any other degree.

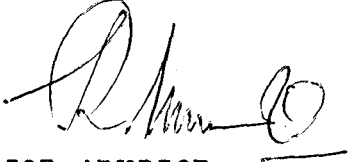
The assistance and help received during the course of investigation have been fully acknowledged.

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MAJOR ADVISOR
(Dr. R.N. Srivastava)

CERTIFICATE - II

This is to certify that the thesis entitled "Molecular Studies on Virus Identification and Serosurveillance of Bovine Herpesvirus type - 1 " submitted by Namrata Pandita to the Haryana Agricultural University, Hisar, in partial fulfilment of the requirements for the degree of Master of Veterinary Science in the subject of Veterinary Microbiology has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.


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To
Dear
Mataji-Pitaji
&
Shubha-Gashaji

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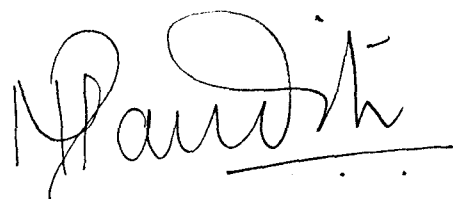
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A handwritten signature in dark ink, appearing to read 'Namrata Pandita', with a horizontal line underneath.

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INTRODUCTION

INTRODUCTION

Bovine Herpesvirus type-1 (BHV-1), an important virus responsible for great economic losses in livestock industry, is prevalent world wide. Even though other wild and domestic animal species are susceptible to this virus, the losses it causes to dairy and beef cattle industry are staggering. BHV-1 has been identified as a major single cause of economic loss in cattle industry in U.S.A. (Yates, 1982). The virus is known for its numerous clinical manifestations viz., rhinotracheitis, conjunctivitis, vulvovaginitis, balanoposthitis, mastitis, abortions and encephalitis. In new born calves it causes alimentary and systemic infections.

In India, with the cattle population of 19.24 crore which is one-sixth of the world, BHV-1 is emerging as a virus of paramount importance. Various studies on BHV-1 show that the exotic and crossbred animals are more susceptible to the disease as compared to indigenous animals. So, with nation wide cross-breeding programme gaining momentum and 'White Revolution' to begin its third phase, this virus attains a more prominent position in the dairy industry of India. Serological survey revealed not only wide spread but also high percent prevalence of BHV-1 in different parts of the country. However, isolation of the virus has been a frustrating experience in many laboratories of the country.

BHV-1 is an enveloped DNA virus placed in the Herpetoviridae family. Like other members of this family, BHV-1 has the enigmatic property to remain latent which is also considered as the mechanism of virus perpetuation in nature. Milk and semen are the most important vehicles for the virus dissemination. The presence of the virus in semen also forms a barrier for free international export of animal and germ-plasm.

With the multiple clinical manifestations shown by this virus, it is not easy, to diagnose the disease on the basis of clinical syndromes alone. Furthermore, to differentiate between the vaccinated and diseased animals on the basis of serology is not straight forward. The detailed epizootiological studies on this virus will eventually unravel the mystery of the virus transmission which could pave the way for formulating an effective control programme. Both attenuated and inactivated vaccines against this disease are marketed abroad. But the animals inoculated with a live vaccine, shed virus upto several months after vaccination. (Gregerson and Wagner, 1985). The studies on inactivated vaccines have also given conflicting results. Thus, emphasizing the need for a safe, efficacious and compatible BHV-1 vaccine. Therefore, research is now directed to find a subunit or recombinant vaccine which will specifically stimulate a strong protective immune response for the elimination of virus from the system. Thus far efforts in this direction have been futile.

Initially, the failure of vaccine was attributed to multiplicity of strains, as in many other viruses. But the virus neutralization and polypeptide patterns did not show any significant differences. Misra et al. (1983) using restriction endonuclease pattern of the genome have suggested to catagorize BHV-1 into 3 strains and 9 sub-strains. However, they were not able to associate any one of the strains with a specific clinical syndrome. Since the information on the genetic and antigenic profiles of BHV-1 isolated from India is 'scanty', it would be of great significance to study the restriction pattern and antigenic structure of the Indian isolates. These studies especially, the antigenic structure would ultimately help to engender effective immunogens for vaccine development and the development of new diagnostic tools suited for India.

Thus keeping in view, the unpalatable economic implications of this virus and with little information on the molecular status of BHV-1 in India, the following studies were undertaken :

1. To carry out the surveillance of BHV-1 in organized cattle and buffalo farms of Haryana state by detection of BHV-1 antibodies in serum samples.
2. To attempt the isolation of BHV-1 in cell culture from clinical cases.
3. To develop and standardize a specific, sensitive, rapid and easy diagnostic test for field use.
4. To compare the Indian isolates of BHV-1 at molecular level and study their antigenic variation/homology, if any.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Introduction :

Bovine herpesvirus type - 1 (BHV-1) is a DNA virus infecting primarily the cattle and buffalo population of the world. The virus is manifested by a variety of clinical syndromes viz., infectious bovine rhinotracheitis, vulvovaginitis, balanoposthitis, conjunctivitis, keratoconjunctivitis, abortions, mastitis, encephalitis and systemic infection.

Infectious bovine rhinotracheitis (IBR) was first noticed in beef feedlots in Colorado, USA, in 1950. It took 4 years to realize the importance of this disease as a major economic problem in the dairy herds and beef feedlots in California and Colorado states of USA (Schroeder and Moys, 1954 and McKercher et al., 1955a). The disease initially had several names such as 'red nose', 'dust pneumonia', 'necrotic rhinitis' or 'necrotic rhinotracheitis' based on the signs and symptoms manifested by this virus in the different parts of the world (Miller, 1955). In Kenya, the reproductive form of disease due to this virus was reported as early as 1938. It was called 'epivag' i.e., causing epididymitis in males and vaginitis in females. But the two disease syndromes were not related for a long time till an antigenic identity was demonstrated in cross neutralization (Gillespie et al., 1959 and McKercher et al., 1959b) and cross protection tests (McKercher, 1963).

In 1955, the US Livestock Sanitary Association coined the name IBR, again based on the clinical syndrome manifested by the virus (McKercher et al., 1955b). It was not until 1973, when the International Committee for the Taxonomy of Viruses (ICTV) named it the Bovid Herpesvirus type - 1 (Roizmann, 1973) on the basis of the family of virus and the host it infects. The nomenclature system was further discussed in the International Committee for the Taxonomy of Herpesvirus (ICTH) in 1978 (Fenner, 1976). Finally, Gibbs and Rweyemamu (1977) adopted a system based on sub-family of Bovidae i.e. Bovine Herpesvirus type - 1 (BHV-1) because the Bovidae family included 128 species. The virus was for the first time successfully isolated in tissue culture by Madin et al. (1956) from the respiratory form of the disease in USA and was identified as herpesvirus.

Clinical manifestations :

Cattle and buffaloes are the primary host of BHV-1 but it may also infect goat, pig, deer and many other wild animals which are less susceptible. The type of disease caused by this virus depends mostly on the route of virus entry. When it invades the upper respiratory tract, it causes rhinitis and tracheitis which may be identified by hyperemic mucosae, copious bilateral nasal discharge which is clear at first and then becomes mucopurulent. There may be drooling of saliva but no mouth lesions. There are lesions on the muzzle of the infected animals which

subsequently become necrotic. As the disease progresses, a diphtheritic membrane forms over the nasal mucosa and in severe cases over the muzzle. There may be rise in temperature (40.5C to 42C), slight anorexia, dullness and in milking animals sharp decline in milk yield. In India, this form has been recently reported (Singh et al., 1989).

On invading the ocular system the virus causes either bilateral or unilateral conjunctivitis or keratoconjunctivitis with profused lacrimation (Abinanti and Plummer, 1961, Quin, 1961, Studdert et al., 1961, Hughes et al., 1964, St. George, 1965 and Timoney and O'Connor, 1971). The animal may become photophobic and epiphora has commonly been observed. The virus has also been isolated from bovine ocular carcinoma in Nevada (Sykes et al., 1962-64 and Taylor and Hanks, 1961) and Argentina (Epstein, 1972).

In the reproductive system the virus manifests as infectious pustular vulvovaginitis (IPV) and balanoposthitis (IPB) (Kendrick et al., 1958 and Bouters et al., 1960). The former is characterized by pustules in vulva and vagina along with mucopurulent discharge and the later with similar lesions on the penile region. Outbreaks of IPB have been described in bulls at the artificial insemination centres (Huck et al., 1961, Knoblauch, 1962 and Straub and Mackle, 1965). The virus has also been isolated from stored semen straws collected from clinically normal bulls (Spradbrow, 1968 and St. George and Philpott, 1972). IPV usually develops 1-3 days after mating and the first

characteristic signs noticed are frequent micturition and tail switching. The vulva becomes oedematous and hyperemic with small pustules on the mucosal surface. These then coalesce to form a yellowish-white fibrinous membrane which when detaches forms ulcers. The animal may show rise in temperature, depression and anorexia. The influence of this virus on bovine fertility is however, controversial.

Although in 1955, Jensen et al. reported that heifers infected with BHV-1 frequently aborted, it was not until 1962 when Ormsbee (1963) isolated BHV-1 from aborted fetus. Abortion usually occurs between 4-7th month of gestation. This is usually accompanied by retention of placenta. The cotyledons are usually blanched and degenerated as the abortion is due to foetal death. In young calves, the invasion of BHV-1 causes systemic infection (Reed et al., 1977).

It was known earlier that BHV-1 may produce mastitis if inoculated into udder (Greig and Bannister, 1965 and Corner et al., 1967). But the virus was isolated from cattle with mastitis in 1974 (Gourlay et al., 1974 and Roberts et al., 1974).

French (1962 a,b) found that the virus isolated from an outbreak of encephalitis in calves in Australia (Johnson et al., 1962) was serologically related to BHV-1. Later it was confirmed in several countries (Bartha et al., 1969, Lomba et al., 1973, Bagdadi and Martin, 1974, Beck, 1975 and

Gough and James, 1975). Affected animals initially show slight incoordination which progresses to ataxia. Periods of depression are followed by those of excitement which are characterized by incoordinated running, stumbling and falling. This is followed by the development of clonic spasms of the neck, leg and lumbar muscles. The animal shows opisthotonus and appears blind. The animal is in coma and dies in 3-4 days after the onset of symptoms. Recovered animals remain blind.

BHV-1 virus :

Structure and physio-chemical properties : The virus isolated from IBR by Madin et al. (1956) was classified as herpesvirus by comparing the in vitro growth and morphology with herpes simplex virus (Armstrong et al., 1961). BHV-1 is a linear double stranded DNA virus (Russel and Cranford, 1964 and Gibbs et al., 1970). The capsid is composed of 162 capsomeres arranged in an icosahedral symmetry. The nucleocapsid of the virus is surrounded by an envelope (Cruickshank and Berry, 1965, Watrach and Bahnemann, 1966 and Bocciarelli et al., 1966). Gibbs et al. (1971) and Talens and Zee (1976) have shown spike like structures on the envelope. The nucleocapsid is formed in the nucleus of the host cell and it acquires the envelope on budding from the nuclear membrane (Jasty and Chang, 1971, Zee and Talens, 1972 and Silva et al., 1973). The virus thus formed is about 200 nm (Watrach and Bahnemann, 1966) with a buoyant³ density ranging from 1.249 to 1.254 g/cm³ in cesium chloride

while it is 1.22 g/cm^3 in potassium tartrate (Talens and Zee, 1976). The nucleic acid has a molecular weight of 84×10^6 daltons, approximately, having 136-139 kbp (Mayfield et al., 1983 and Seal et al., 1985) and a buoyant density of $1.73\text{-}1.732 \text{ g/cm}^3$ in cesium chloride (Russel and Crawford, 1964, Plummer et al., 1969, Graham et al., 1972 and Ludwig, 1972). The virus is heat labile. It is inactivated within 21 min. at 56C, 15 h at 37C, 15 days at 22C, one month at 4C and 9 months at -60C. Lyophilization causes fall in titre by 1 log (Griffen et al., 1958, Snowdon, 1964, Hahnefeld et al., 1963 and Schudel et al., 1982). The virus survives best in aerosols at low (20-40%) relative humidity. Presence of 0.5M magnesium chloride makes virus stable in water. BHV-1 is stable at pH 6.0-9.0 but labile to pH 4.5-5.0. Because of the lipid envelope, the virus is extremely susceptible to lipid solvents such as ethyl ether, acetone, ethyl alcohol and chloroform (Stevens and Groman, 1963). Straub (1965) tested the susceptibility of BHV-1 to various disinfectants and found that the virus was rapidly inactivated by 0.5% sodium hydroxide, 0.01% sodium chloride, 1% chlorinated lime, 1% phenolic derivatives and 10% lugol's iodine.

The viral proteins and genome : Sklyanskaya et al. (1977) reported 18 structural polypeptides with molecular weight ranging from 29 to 250 kilo daltons (KDs), of which 8 were glycosylated. Certain of these polypeptides correlate with the data of Misra et al. (1983) and Bolton et al. (1983).

However, lack of quantitation and poor resolution of their gel system made a specific comparison difficult. Pastoret et al. (1980) studied the protein profile of different strains of BHV-1 from different origins (respiratory and reproductive). The study revealed a total of 21 polypeptides with molecular weight varying from 13 KD to 275 KD of which 10 were glycosylated in BHV-1 isolates from both origins. But they differed in 3 polypeptides i.e., VP12 and VP13 were of higher molecular weight (68 and 66 KDs) in IPV isolates while VP7 was larger in IBR isolate (99 KD). Misra et al. (1981) used purified radiolabelled virus both 'empty' and 'full' for electrophoretic analysis. The study revealed the presence of 25 structural virus polypeptides of which 11 were glycosylated. At least 15 non-structural polypeptides were also identified which were absent in purified preparations. Protein analysis by 2-D electrophoresis further revealed that 5 of these bands of SDS-PAGE might contain 2 or 3 proteins. They, thus suggested that BHV-1 contained more than 25 polypeptides. Confirming this, Bolton et al. (1983) using purified virus (by rate zonal and isopycnic centrifugation in potassium tartarate gradient) reported the presence of 33 polypeptides. The gel was scanned spectrophotometrically. The molecular weight of the polypeptides ranged from 13 to 275 KD. The study suggested that VP8 and VP13 constitute the major outer surface antigen which compose the envelope spikes. Four virus-specified glycopolypeptides with molecular weights 69-75 KD, 77-81 KD, 82-92 KD and 108-115 KD were shown to induce serum

neutralizing antibodies (Collins et al., 1984 and Lum and Reed, 1986). The 69-75 KD and 82-92 KD glycoproteins of this study corresponded to VP13 and VP8 of Bolton (1983) and GVP11 and GVP9 of Misra et al. (1981), respectively, while the 77-81 KD glycoprotein did not resemble any earlier known polypeptides. Trepanier et al. (1986) used PAGE for strain differentiation of BHV-1 but found only minor differences which were not appreciable.

Thus, as the protein profile did not seem to have a good prospect for strain differentiation, the genomic profile was probed into. Engles et al. (1981) reported significant differences between the restriction endonuclease (RE) cleavage patterns of the IBR and IPV isolates of BHV-1. In contrast, Misra et al. (1983) in their analysis of 116 BHV-1 isolates reported that the differences in cleavage patterns were not associated with the disease syndromes manifested by BHV-1. According to the cleavage pattern, the authors divided BHV-1 into 3 strains (I, II and III) and 9 sub-strains. Seal et al. (1985) showed 95% genetic homology between the BHV-1 isolates from different syndromes. Thus suggesting that the differences in the RE pattern may be due to point mutation(s) which resulted in loss or gain of RE site. Considering such high degree of DNA homology amongst various isolates, the reason for differences in clinical manifestation is not well understood and may be due to the route of infection (Misra et al., 1983).

Immune Response :

A variety of specific immunological mechanisms have been shown to be effective in neutralizing herpesvirus or destroying the infected cells (Gibbs and Rweyemamu, 1977). A complicating factor with this virus is its ability to set up persistent infection which the immune system fails to eliminate. However, BHV-1 induces both humoral and cell mediated immune (CMI) response or combination of both (Gibbs and Rweyemamu, 1977). It is generally expected that the humoral immune response is responsible for preventing reinfection whereas CMI is more important for the recovery from this viral infection (Kahrs, 1977). The antibody production as detected by neutralization is detected only after recovery whereas the CMI and interferon production occurs earlier and is more closely correlated to the time of recovery (Rouse and Babiuk, 1974, 1975, 1976 and Rouse et al., 1976).

Even though the CMI contributes more to the recovery from infections, antibody response to BHV-1 has received more attention for the diagnostics and epidemiological studies. Neutralizing antibodies to BHV-1 can first be detected in serum of affected cattle at about 8th day post infection (PI) (Davies and Carmichael, 1973, Rouse and Babiuk, 1974) and persist upto 5.5 years after infection (Chow, 1972). Passive immunity is transferred to the calf in the colostrum and can be detected in calf till it is 4-6 months of age (Straub, 1969). Neutralizing

epitopes have been identified on different glycoproteins (Collins et al., 1984 and Lum and Reed, 1986). Trepanier et al. (1986) identified a neutralizing epitope on the 90 KD hemagglutinin which cross neutralized with all the 14 strains they tested. In a functional and topographical analysis of epitopes of major antigenic proteins of BHV-1, 3 out of 12 monoclonal antibodies studied, with the strongest neutralizing activity were those which recognized a band at 70 KD in western blot (Scott et al., 1988 and Hughes et al., 1988). The gIV (71 and 140 KDs, the monomeric and dimeric form) is a BHV-1 induced early glycoprotein. Hughes (1988) described three distinct neutralizing antigenic domains on it gIV as I, II and III. Domain I comprises of 2 overlapping epitopes while domain II was represented by a single monoclonal antibody and domain III was made up of a complex of 4 identical or overlapping epitopes designated as a, b, c and d. He further suggested that domain I of gIV may be involved in the penetration of the virus into the host cell. It is also accessible to the immune system and can participate as a target for immunocytolysis at an early stage by antibody dependent cell cytotoxicity (Misra et al., 1982). Thus gIV has an added importance of being an early virus induced glycoprotein expressed on the surface hence it is the first viral gene product recognized by the host's immune system. Secondly, in case of reoccurrence/reactivation it will again be the first viral target, and these may also be the only viral targets available to the immune

system in latency and individuals treated with virostatic drugs. So, gIV was probed for strong neutralizing antibodies.

BHV-1 is a cell associated virus and spreads through intracellular bridges (Christian and Ludovici, 1971), thus it does not enter the extracellular environment till infection is set. However, the antibodies play an important role in limiting the virus through the mechanism of antibody dependent cell cytotoxicity (ADCC). The infected cells express viral antigens on their surface (Roane and Roizman, 1964 and Babiuk et al., 1975). The antibody enhances the cytotoxicity of the infected cells (Babiuk et al., 1975, Rouse et al., 1976a, 1976b, 1977a and 1977b).

The non-specific immunity by the macrophage has little role to play in BHV-1 as the virus is able to replicate in them (Rouse and Babiuk, 1978). Thus, the macrophage might even help in spreading infection. Contrary to this, Srivastava and Jan (1982) have shown non-specific antiviral activity of bovine leukocyte against BHV-1 in vitro. However, there has been a great deal of evidence to support the idea that specific CMI is the most important mechanism of recovery from cell associated viruses. Although a variety of cellular mechanisms have been reported to function against herpesvirus infected cells, cytotoxic T cells (CTL) are suspected to be of major importance (Oakes, 1975 and Sethi et al., 1980). This mechanism can be

enhanced by the presence of neutralizing antibodies and complement. The viral antigens were detected on cell surface 6 h PI and spread after 10 h PI. Thus, if cells are destroyed before 10 h PI virus gets no time to be released (Misra et al., 1982). Cytotoxicity against BHV-1 infected cells (Rouse and Babiuk, 1977) was confirmed by demonstrating generation of proliferative population of CTL cells using the limiting dilution frequency analysis (Splitter and Eskra, 1986; Miller-Edge and Splitter, 1986a and 1986b).

Splitter et al. (1988) have provided evidence that CTL have antigen-specific role in immune response of cattle against BHV-1. Whether the MHC restriction is class I or II or both is not known at present. These CTL may further serve as effector cells in identification of glycoproteins useful in recombinant vaccine preparation. Recently, a suppressor cell population has been shown to be activated by BHV-1 (Miller-Edge and Splitter, 1986b). Carter et al. (1989) demonstrated suppression of T-cell proliferation by BHV-1 which was the result of a non-productive BHV-1 infection.

Since the control of BHV-1 is based largely on vaccination, the safety and potency of the commonly used live attenuated and killed vaccines have been questioned (Zuffa et al., 1979 and 1982, Rossi et al., 1982, Mann et al., 1983; Kita et al., 1983 and Nettleton et al., 1984). Therefore, the use of subunit vaccine consisting of the

immunodominant epitopes would be a viable alternative. This would also solve the problem of latency due to vaccinated strain. A considerable progress has been made towards production of subunit vaccines against BHV-1 due to availability of recombinant DNA technology (Kleid et al., 1981, Bittle et al., 1982 and Cohen et al., 1984).

This needed more information regarding the polypeptides and mechanisms involved in immune responses against BHV-1. Studies on the topography of glycoproteins have revealed 4 unique immunogenic glycoprotein viz., gI, gII, gIII and gIV (Misra et al., 1982, van Drunen Littel-van den Hurk et al., 1984, van Drunen Littel-van den Hurk and Babiuk, 1986 a). The gI and gIV protein have been submitted to amino - terminal sequence analysis (L'Italien and Zamb, 1988). In addition, the genes for gI, gIII and gIV have been identified and sequenced. It was found that gI, gIII and gIV of BHV-1 correspond to gB gC and gD of HSV respectively (Zamb, 1988). Purified gI and gIII stimulate the production of monospecific antibodies which neutralize viral infectivity and mediate immune lysis of virus-infected cells (van Drunen Littel-van den Hurk and Babiuk, 1985). These individual glycoproteins also stimulated antibody production which protected cattle infected with whole virus. Thus, suggesting their importance in mediating protective immune response against BHV-1 (van Drunen Littel-van den Hurk and Babiuk, 1986b). Babiuk et al. (1987) immunized animals with subunit vaccine using the

glycoproteins gI, gII, gIII and gIV either individually or in combination and showed protection against the disease but not infection.

Epizootiology :

The literature available on the epizootiology of BHV-1 induced disease syndrome is very scanty. The disease has been reported from most of the countries (Gibbs and Rweyemamu, 1977). The virus naturally infects cattle, buffaloes, goats, sheep, pigs and wild animals whereas experimental infection may be set up in rabbit and ferret. The virus is reported to survive better at lower relative humidity i.e., 20-40% (Gibbs and Rweyemamu, 1977). The presence of magnesium chloride (0.5 M) in water stabilizes the virus. Studies on the seasonal preference revealed that the number of out (break)s during January to April were more as compared to other months (Epidemiology Unit Report, Weybridge, 1979). They also suggested that housed adults of both dairy and beef feedlot were more susceptible and there were fewer out (break)s in young calves. The morbidity rate varies from 20-100% depending on the management and sanitation. Studies over the years have shown a steady increase in the number and severity of outbreaks (Epidemiology Unit Report, Weybridge, 1979).

Studies done on the transmission of BHV-1 are meager. No intermediate vector responsible for its transmission has been identified. However, mechanical transmission by insects and fomites has been suggested

(Hayne and Johnston, 1964 and Collings et al., 1972). Studies indicated that milk and semen were the two most important vehicles of virus dissemination (Gibbs and Rweyemamu, 1977).

Another area of epizootiological interest is the perpetuation of BHV-1 in nature. No reservoir host for BHV-1 has been identified as yet. However, the enigmatic property of latency seems to play the major role in viral persistence and perpetuation. In the infected animals the virus remains in an undetectable form in the ganglionic neurons (Stevens, 1978). In these latent animals BHV-1 can be reactivated by treatment with corticosteroids or immunosuppressants (Sheffy and Rodman, 1973, Davies and Duncan, 1974 and Kretzschmar et al., 1983). BHV-1 has been suggested to become latent following vaccination (Pastoret et al., 1982, Rossi and Kiesel, 1982 and Straub, 1984). Thus, highlighting the importance of knowing the strains circulating in the populations before the herds are vaccinated with another live vaccine strain.

Another mechanism that usually aids persistence and perpetuation of a virus in nature is the multiplicity of strains and sub-strains like in FMDV. But virus cross neutralization, polypeptide pattern and antigenic patterns of BHV-1 did not show any significant differences amongst BHV-1 strains (Pastoret et al., 1980, Misra et al., 1981, Gregersen et al., 1985, Metzler et al., 1985 and Trepanier et al., 1986). Thus, Misra et al. (1983) used restriction

endonuclease pattern of the genome to catagorise BHV-1 into 3 strains and 9 sub-strains. However, to associate any one of them with a specific clinical syndrome was not possible.

Status of BHV-1 infections in India :

In India, this disease was first reported from an outbreak of keratoconjunctivitis among crossbred calves (Mehrotra et al., 1976). Since then BHV-1 outbreaks have been reported from many states of the country. Samal et al. (1981) reported approximately 56.5% of the total serum samples tested and 65.2% of the samples with history of abortions, positive for BHV-1 antibodies by indirect hemagglutination (IHA) . Sulochana et al. (1982) surveyed occurrence of BHV-1 in Kerala and reported higher percent positivity in aborted cases. Tongoankar et al. (1986) carried out a study on the cause of abortions which revealed that 46% aborted animals were positive for BHV-1 antibodies in comparison with 11.9% by bluetongue antibodies and only 5.3% each for Chlamydia and Brucella. Mallick et al. (1986) collected a total of 1984 samples from different states of the country namely, Andhra Pradesh, Karnataka, Orissa, West Bengal, using IHA and serum neutralization test (SNT). The presence of BHV-1 antibodies was detected in 73.26% of exotic animals, 63.46% of crossbred, 62.01% of indigenous cattle and 64.24% in buffaloes. The overall percent seropositivity was 66.08. Singh et al. (1986) stated that on an average 32.34% of the dairy bulls were positive for BHV-1 antibodies. Seropositivity was higher in bulls

maintained in the southern region of the country, exotic animals than indigenous and adults as compared to young ones. In a study carried out on bovines by Satyanarayana and Suribabu (1987) in Andhra Pradesh, 88.81% exotic, 76.92% crossbred, 81.48% indigenous cattle and 70.47% buffaloes were seropositive by IHA. The overall seropositivity reported by them was as high as 77.46%.

Mehrotra (1979) isolated BHV-1 from an aborted crossbred cow for the first time in India. Subsequently the virus was isolated from other parts of the country from semen, aborted material/foetus, respiratory tract and conjunctivitis (Singh et al., 1986, Mehrotra et al., 1977, Misra and Misra, 1987 and Singh et al., 1989). Misra and Misra (1987) have shown BHV-1 association with infertility and repeat breeding. Mehrotra et al. (1987) have also shown the reactivation of BHV-1 following steroid treatment in vaccinated herds.

Diagnosis

Any disease control programme has a prerequisite of diagnosis at the earliest. The serological assays for antibodies to BHV-1 have been widely used for the diagnosis of acute infections, epidemiological studies and the detection of latent carriers in relation to control schemes and international trade. Gibbs and Rweyemamu (1977) have reviewed the diagnostic tests then available for BHV-1. These included the conventional clinical diagnosis based on signs and symptoms but as the virus has numerous clinical

manifestations, diagnosis is difficult and required laboratory confirmation. The laboratory diagnosis includes isolation and characterization of the virus. The characteristic cytopathic effect (CPE) includes rounding with refractile syncytia formation. As infection progresses strands of cytoplasm are seen linking the infected cells. Intranuclear viral inclusions of Cowdry type A can be identified in infected cells both from biopsy and cell culture (Collings, 1972). Electron microscopy (EM) is another rapid reliable method of diagnosis of BHV-1 except the fact that all herpesviruses are indistinguishable by EM (Gibbs and Rweyemamu, 1977).

In addition, there are various serological techniques available for the retrospective diagnosis of BHV-1 infection. These techniques, devised to detect antibodies against BHV-1, include various serum neutralization tests (Dawson, 1962, Mohanty and Lillie, 1965, Greig, 1969, House and Baker, 1971 and Singh et al., 1984), passive hemagglutination test (Whitman and Hetrick, 1965, Kirby et al., 1974, Trepanier et al., 1985), agar gel diffusion test (Kaminjolo and Gicho, 1972), complement fixation test (Wellemans and Lennen, 1973) and an indirect fluorescent antibody test (Assaf et al., 1975). Serum neutralization test is widely used as a standard test for BHV-1 antibody detection although it is less sensitive (Rossi and Kiesel, 1974). This test was modified to increase its sensitivity by adding complement (Rossi and Kiesel, 1974 and Potgieter,

1975) and prolonging the neutralization reaction (Bitsch, 1978). Payment et al. (1979) reported enzyme linked immunosorbent assay (ELISA) to be most sensitive and reliable (Herring et al., 1980, Bolton et al., 1981 and Riegel et al., 1987). Edwards et al. (1986) conducted a study to evaluate 5 serological tests for detection of antibodies to BHV-1. The sensitivities of ELISA and VNT (24 h neutralization reaction) were similar in contrast to PHA and VNT (one h neutralization reaction) which failed to detect low titers of antibody. Smith et al. (1987) reported that immuno-peroxidase (IP) on fixed tissue was more sensitive for detecting antibodies against BHV-1 as compared to fluorescent antibody test (FAT).

The major limitation in the diagnosis of BHV-1 is latent infection. Rock et al. (1986) detected BHV-1 RNA in the trigeminal ganglia of latently infected rabbits by in situ hybridization. The viral RNA appeared to be restricted to the nucleus of the latently infected cells. Thus, indicating that BHV-1 genome is transcriptionally active in the ganglionic neurons during latent infection. Subsequently it was found that there is specific viral transcription which is responsible for establishing and maintaining latent BHV-1 infection (Rock et al., 1987 and Stevens et al., 1987).

MATERIALS & METHODS

MATERIALS AND METHODS

All the media and reagents used in this study were prepared in deionized triple glass distilled water (TGDW).

COLLECTION OF SAMPLES :

Materials and Reagents :

1. Heparin stock solution : A stock heparin (Sigma Chemicals, USA) solution of 100 IU/ml was prepared in sterilized TGDW and seitz filtered (EKS pads, 0.45 u). This was used for collection of blood samples for the virus isolation at the rate of 1 ml heparin stock solution per 5 ml of blood. The stock solution was stored at 4C and checked for sterility.
2. Antibiotics stock solution : A stock solution of penicillin (10,000 IU/ml), streptomycin (10,000 ug/ml) and mycostatin (10,000 IU/ml) in Hank's balanced salt solution (HBSS) was made. Mycostatin is insoluble in HBSS. Therefore, it was triturated in absolute ethanol in mortar and pestle separately, and then centrifuged at 2,000 rpm for 15 min. The supernatant was added to HBSS to make the total volume 100 ml. This was filtered through seitz filter. Sterility was tested and the solution was stored in small vials at -20C. Of this stock solution, 1% was added to all media before use.

3. Sodium bicarbonate solution : A solution of sodium bicarbonate (3.7%) was prepared in TGDW, dispensed in small vials and sterilized by autoclaving at 15 lb pressure for 15 min.

4. Transportation medium (TM) : Minimum essential medium (MEM) in Hank's base with non essential amino acids (NEAA) (Centron Research Laboratories, Thane) was prepared in TGDW having 0.3% tryptose phosphate broth (Hi-media, Bombay) and 0.02% L-glutamine (Sisco Research Laboratories, Bombay). This was then sterilized by seitz filtration and tested for sterility. The media was supplemented with 1% stock antibiotic solution just before use. The pH was adjusted to 7.2 with sodium bicarbonate .

Method :

Heparinized blood samples were collected within the week of abortion for virus isolation. Pieces from the lung, liver and cotyledons of aborted foetus were also used for BHV-1 isolation. Nasal and vaginal swabs were collected in TM. All samples collected for virus isolation were carried on ice to the laboratory and processed immediately.

For serological survey, serum samples were collected from aborted and incontact healthy animals (cattle and buffaloes) from various organized farms in Haryana. These farms were Government Livestock Farm (GLF) Sector I, II and III, Hisar; Central Institute for Research on Buffaloes,

Hisar; Haryana Agricultural University Animal Farm, Hisar; National Dairy Research Institute, Karnal; Model Exotic Farm, Bhiwani and Military Animal Farm, Ambala. Serum samples were also collected from a bull breeding farm, Indo-Dairyherd Improvement Centre, Rohtak. The serum samples were stored at -20C until screened.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) :

Serological survey was done using ELISA (Flow Laboratory Diagnostic Kit, USA) according to the procedure prescribed by the manufacturers.

Materials and Reagents :

All the reagents were provided in the kit viz., BHV-1 antigen coated plates, standard positive and negative serum against BHV-1, anti-bovine horse radish peroxidase (HRPO) conjugate, hydrogen peroxide, 22' amino bis 3-ethyl benzthiozoline sulphate (ABTS) substrate and phosphate buffer saline Tween- 20 (PBST) as a 10x solution to be diluted in TGDW.

Method :

A constant dilution (1:40) of all the test samples and standards were made in 1x PBST. The coated plates were incubated with 100 ul of test serum in each well and the standards in a moist chamber at 37C for 1 h. The plate was then thoroughly washed in PBST (three times) and was incubated with 100 ul of conjugate in each well at 37C for 1 h. Again the plate was properly washed in PBST (six

times). Finally, substrate ABTS (100 ul) with hydrogen peroxide added just before use, was added to each well and incubated at 37°C for 5 min. to allow enzyme action. At the end, 100 ul of 0.1% hydrofluoric acid (pH 3.3) was used to stop the enzyme reaction and the optical density was recorded at 405 nm wave length using an ELISA reader (Titertek Multishan MCC-340).

DOT IMMUNOBINDING ASSAY (DIA) :

DIA was standardized to detect BHV-1 antibodies in serum under field condition. The standard procedure was followed (Hawkes, 1986).

Materials and Reagents :

1. Dip stick: Nitro cellulose paper (pore size 0.45 u) 0.7 x 0.7 bound to plastic strips (7 x 75 mm) (East Punjab Manufacturing Co., Hisar) were used .
2. BHV-1 antigen : This was prepared by concentrating IVRI stock virus 10 fold by dialysis against PEG 6000. This was used for coating the dip sticks.
3. Conjugate : Anti-cow HRPO conjugate (Sigma Chemicals, USA) was used in 1:200 dilution.
4. Substrate : The substrate was prepared in citrate buffer (pH 5.0) using 0.05% diaminobenzidine (DAB) (Sigma Chemicals, USA) and 1 ul/ml hydrogen peroxide (30%) just before use.

5. Phosphate buffer saline Tween- 20 (PBST) : PBS (pH 7.2) containing 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate and 8.1 mM disodium hydrogen phosphate in TGDW. To this, 0.05% Tween-20 (Sigma Chemicals, USA) was added.

6. Blocking buffer, pH 7.2 : Five percent skimmed milk powder (Lactogen) was made in PBST (pH 7.2). It was kept at 37C for 15-20 min. to dissolve the milk powder.

7. Citrate buffer, pH 5.0 : Dibasic disodium hydrogen phosphate anhydrous (0.2 M) 25.7 ml and 24.3 ml of 0.1 M citric acid (Hi-media, Bombay) were added to make 100 ml in TGDW. The pH was adjusted to 5.0.

Method :

The dip sticks were washed in distilled water and dried before use. Then approximately 2 ul of BHV-1 antigen was deposited at the center of NCP on each dip stick, with the help of a capillary tube. These were allowed to dry at 40C. The unsaturated sites on the NCP were blocked by blocking buffer for 2 h at 37C. The unbound proteins were washed off by thoroughly rinsing in PBST (3 change). This was followed by incubating the dip sticks in test serum for 1 h at 37C. The unbound serum was completely removed by vigorous washing in PBST (3 changes). Thereafter, the dip sticks were immersed in conjugate and incubated for 30 min. at 37C. The dip sticks were then thoroughly washed in 3 changes of PBST and immersed in DAB substrate for 1 min.

during which brown colour developed at the site of antigen-antibody reaction. The sticks were washed to stop reaction, air dried and stored.

CELL CULTURE :

Materials and Reagents :

Madin Darby bovine kidney (MDBK) cell line was procured from the Department of Zoology, University of Pune, Pune. It was used for virus isolation, characterization and propagation.

1. Growth medium (GM) : The growth medium was similar to TM except that GM was supplemented with 10% seitz filtered and inactivated precolostral bovine calf serum and 1% stock antibiotic solution just before use. The pH was adjusted to 7.2 with sodium bicarbonate.
2. Maintenance medium (MM) : The composition was similar to the GM except that precolostral bovine calf serum was added at the rate of 2% instead of 10%.
3. Trypsin-phosphate-glucose-versine solution (TPGV) : The stock solution was made by dissolving 2% trypsin 1:250 (Difco, Bombay), 1% glucose (AnalaR, Bombay) and ethylene diamine tetra acitic acid (EDTA) 4% (Sigma Chemicals, USA) in sterilized PBS by stirring on a magnetic stirrer for 1 h at 4C. It was then centrifuged at 2,000 rpm for 10 min. in a refrigerated centrifuge (4C). The supernatant was sterilized by seitz filtration. The pH of the stock ^{soln.} was

adjusted to 7.2 and was tested for sterility. This was dispensed in 20 ml vials and stored at -20C.

To prepare working solution, the stock solution was diluted 20 times in PBS (pH 7.2) to make a 0.1% solution.

4. Cell preservation solution : Dimethyl sulpha oxide (DMSO) 10% (Sigma Chemicals, USA), inactivated precolostral bovine calf serum 20% and antibiotics 1% (stock solution) was made in MEM (Hank's with NEAA). The pH was adjusted to 7.2 with sodium bicarbonate, sterilized by seitz filtration and stored at -20C in small vials.

Method :

Cell culture : A confluent monolayer was washed with medium and trypsinized with TPGV (0.1%) for 1 min. After complete decanting of TPGV, the cell culture bottles were kept at 37C for 2-3 min. Then GM was added and the cells were separated by gentle but forceful pipetting to make a uniform cell suspension. This was then used as seed for sub-culture. The split ratio used was 1:4. The seeded bottles, test tubes or Rous flasks were incubated at 37C. A complete monolayer appeared after 48 h.

Preservation: For preservation of cells, the cell suspension was made in minimum amount (10 ml) of MEM, after the trypsinization. This was then centrifuged at 4C for 10 min. at 2,000 rpm. The clear supernatant was removed and the cells were suspended in 3 ml of cell preservation solution.

This was equally dispensed in two sterile storage vials (1.8 ml Costar, USA) and sealed with wax. These vials were stored at -80C.

STOCK VIRUS :

Materials and Reagents :

Two BHV-1 isolates National Dairy Development Board (NDDB) (6 passage) and Indian Veterinary Research Institute (IVRI) isolate (29 passages) were obtained from the National Dairy Development Board, Anand. These were further passaged in MDBK upto a total of 10 passages and 33 passages, respectively, to make seed virus.

Method :

MDBK cells were grown in Roux flasks. On attaining a confluent monolayer the GM was removed and cells infected with seed virus (5 ml). The flasks were then incubated at 37C for 2 h for adsorption of virus during which flasks were regularly shaken to rinse the monolayer with the inoculum. Thereafter, the inoculum was decanted and 80 ml of MM was added to each flask. These infected flasks were incubated at 31C (Scott et al., 1988).

The culture medium was harvested when > 90% of cells showed rounding and detachment. These were freeze-thawed thrice to release cell associated virus and stored at -20C till further use. Extensive CPE (> 90%) was generally observed between 60-72 h PI.

Titration of the stock viruses : Infectivity titre of the stock virus was determined in MDBK cells grown in tubes. A ten fold serial dilution (10^{-1} to 10^{-9}) of each of the two virus strains was prepared in MM, separately. A set of three tubes were infected with 0.1 ml of each dilution of each of the virus, after decanting the GM. These were then incubated at 37C for 2 h to allow adsorption of the virus particles. The inoculum was then decanted and 1.5 ml of MM was added to each of the tubes and incubated at 31C. They were examined daily for 4-5 days for appearance of CPE. The infectivity titre was calculated by the method of Reed and Muench (1938).

VIRUS PURIFICATION AND CONCENTRATION :

The virus was concentrated by ultracentrifugation and purified by passing through a 40% sucrose cushion (Scott *et al.*, 1988).

Materials and Reagents :

1. Tris-saline-EDTA (TNE), pH 7.6 : Sodium chloride (0.1 M), Tris-HCl (0.01 M) and EDTA (0.001 M) was made in TGDW. The pH was adjusted to 7.6. It was autoclaved and stored at 4C.

2. Tris-EDTA buffer (TE), pH 7.4 : The buffer constitutes of 0.01 M Tris-HCl and 0.001 M EDTA in TGDW. The pH was adjusted to 7.4 and stored at 4C.

3. Sucrose solution (40%) : Sucrose (40%) was dissolved in TNE buffer and boiled with activated charcoal (5-7%) for 10 min. On cooling this was filtered through a charcoal pad.

Method :

The stock virus was centrifuged at 6,000 rpm for 30 min. at 4C (Sorvall LRC-SB) to remove cell debris. The debris was washed in 5 ml of PBS and centrifuged again at the same rpm. The supernatant was pooled for pelleting the virus by centrifuging at 30,000 rpm for 1.5 h at 4C (Beckman L8-M using Ti-65 rotor). The virus pellet was dissolved in 500 ul of PBS. To purify this virus, it was passed through a 40% sucrose cushion by centrifuging at 40,000 rpm for 1.5 h at 4C (Beckman L8-M using SW-65 rotor). The supernatant was decanted and sides of the tubes were dried with filter paper. The virus pellet thus obtained was dissolved in TE buffer for DNA isolation and sample buffer for protein and antigenic profile.

SODIUM DODECYL SULFATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) :

Materials and Reagents :

1. Stacking gel buffer, pH 6.8 : A solution of 0.125 M Tris-HCl (Sigma Chemicals, USA) was prepared in TGDW. The pH was adjusted first with concentrated hydrochloric acid (HCl) and then with 1N HCl. To this was added 0.1% sodium dodecyl sulphate (SDS) (Sigma Chemicals, USA) and filtered through Whatman filter No. 1. This was stored at 4C.

2. Running gel buffer, pH 8.8 : A 0.375 M Tris-HCl solution was made in TGDW. The pH was adjusted with 1N HCl. It was then filtered through Whatman No. 1 and stored at 4°C till further use.
3. Electrode buffer (EB), pH 8.3 : This buffer consisted of 0.025 M Tris-HCl and 0.192 M glycine (Sigma Chemicals, USA) in TGDW. SDS was added to a final concentration of 0.1%.
4. Stacking gel solution : Acrylamide (Sisco Research Laboratories, Bombay) 3%, bis acrylamide (Sisco Research Laboratories, Bombay) 0.356% and SDS 0.1% were dissolved in stacking gel buffer and the solution filtered through Whatman No.1 filter paper. Just before casting, freshly prepared 10% ammonium persulphate (APS) (Merck, Bombay) at 250 μ l/100 ml and then tetra methylethylenediamine (TEMED) (Sigma Chemicals, USA) at 25 μ l/100 ml were added.
5. Running gel solution : Acrylamide (10%), bis acrylamide (0.356%) and SDS (0.1%) were dissolved in running gel buffer and was filtered through Whatman No.1. Freshly prepared 10% APS (250 μ l/100 ml) and TEMED (25 μ l/100 ml) were added just before use.
6. Sample buffer, pH 6.8 : This consisted of SDS 4%, 2-mercapto ethanol (2-ME) 5% (Merck, Bombay), glycerol (Galaxo, India) 10% and bromophenol blue (Sigma Chemicals, USA) 0.005% in stacking gel buffer.

7. Staining solution : The staining solution contained 0.1% Coomassie blue (R-250), 41.7% methanol (Qualigens, Bombay) and 16.7% acetic acid (Qualigens, Bombay). The dye was dissolved in methanol. Then acetic acid and water were added to make appropriate concentrations. It was filtered through Whatman No. 1 and stored in coloured bottles at room temperature.

8. Destaining solution : The destaining solution for gel consisted of 5% acetic acid and 7% methanol while for nitrocellulose paper (NCP) 2% acetic acid and 90% methanol in TGDW.

Method :

The slab gel SDS-PAGE was carried out by standard technique (Zingales, 1984). This was a modification on the discontinuous buffer system of Laemmli (1970).

Sample Preparation : The partially purified virus pellet was directly dissolved in sample buffer. The suspension was kept for 5 min. in boiling water bath. Then it was allowed to cool.

Assembly of the slab gel apparatus : The slab gel is prepared between two glass plates which were perfectly clean. Insert spacer (1 mm) and seal edges with tape. Place this in casting apparatus.

Preparation of gel : To the running gel solution, freshly prepared APS and TEMED were added just before casting.

The solution was mixed thoroughly and gently poured between glass slabs in the casting apparatus upto about 2.5 cm below notch. The running gel was gently overlayed with water to achieve an even surface. The gel was allowed to set for 40 min.

Thereafter, freshly prepared APS and TEMED were also added to the stacking gel solution and mixed. The layer of water on the running gel was removed, the gel was washed with stacking gel buffer and the stacking gel solution was poured. A comb was then inserted gently to avoid air bubbles. This was kept for 30 min. to set the gel.

Electrophoresis run : After the gel was set, the comb was removed and the slab was fixed in the electrophoretic equipment (Pharmacia, Sweden) with EB. A 15 min. run at 20 mA was made before loading the samples. After loading the samples and markers (Pharmacia, Sweden), electrophoresis was carried out at 30 mA/slab (14x18 cm) for 15 min. followed by 45 mA/slab (14x18 cm) run, for 3 to 4 h.

Staining and destaining of gel : When the gel was removed from in between the glass slabs, the stacking gel was removed and the distance covered by tracking dye was marked. The gel was then left in the staining solution for 5 h.

After 5 h the gel was destained with regular changes of destaining solution at 37°C. On complete destaining the bands visualized were drawn and gel photographed.

WESTERN BLOTTING :

Western blotting was done following the method of Gershoni and Palade (1983). The procedure involves three steps :

Step 1. The viral proteins were separated by SDS-PAGE.

Step 2. These separated polypeptides were transferred onto the NCP electrophoretically, while maintaining their spacial relationships.

Step 3. The NCP was treated with standard antiserum and the presence of the bound antibodies was detected by an enzyme which broke down the substrate to give colour reactions.

Materials and Reagents :

1. Transfer buffer (TB), pH 8.3 : Similar to electrode buffer for SDS-PAGE with additional 20% methanol but no SDS.

2. Phosphate buffer saline Tween-20 (PBST), pH 7.2 : PBS was prepared as described earlier (DIA section). To this 0.3% Tween-20 was added for quench buffer (QB) and 0.05% for wash buffer (WB).

3. Standard antiserum : a) Standard BHV-1 antiserum (Cambridge, UK), raised in rabbit was used at 1:100 dilution in PBST. b) A monoclonal antibody against the neutralizing epitope on the 90 KD hemagglutinin protein was procured from Kansas State University, Manhattan, USA. This was used in a dilution of 1:500 in PBST.

4. Conjugate : Both anti-rabbit and anti-mouse HRPO conjugates were obtained from Sigma Chemicals, USA. A dilution of 1:200 in PBST was used.

5. Nitro cellulose paper (NCP) : The NCP was imported from Sigma Chemicals, USA.

The blocking buffer, citrate buffer and substrate (DAB) were prepared as for DIA. In addition to these reagents, the reagents required for SDS-PAGE were also required.

Method :

For the antigenic profile two gels were loaded and run to separate the polypeptides by SDS-PAGE as described. Just before PAGE was over, the NCP were trimmed to fit the gel with excess at ends for labelling. The NCP with the rough surface up, were washed in distilled water. Then they were transferred to TB for 10 min. Filter paper pads (Whatman 3) were also wet in TB.

Blotting : When the PAGE run was over, the gel assembly were removed. Then the spacers and top plates were removed. In a tray full of TB, the gel were transferred onto the filter paper pads avoiding distortion. The gels were covered with the NCP, dull side in contact with the gel. The NCP overhang were marked with a pencil for orientation. On this were placed the second filter paper pads and the two sandwiches were placed in the cassettes. When the cassettes were placed in the transfer apparatus the order of layers were anode-filter paper pad-NCP-gel-filter pad-cathode.

The blotting was run at 12 V overnight at 4C followed by 24 V run for an hour before shutting off.

Treatment of blots : When the blots were ready, the cassettes were removed and placed with the anode side up. The top layers were removed and the NCP (with dull side up) were placed in QB for 1 h at the room temperature with agitation (50 rpm). The lanes with molecular weight markers of both blots were cut, marked for identification and stained with Coomassie blue to assess transfer.

The NCP blots were then placed in the blocking buffer to block the unoccupied sites for 2 h, with shaking (50 rpm). Subsequently, the blots were washed in 3 washes of WB for 20 min. each. Then each blot was marked for identification as one was treated with polyclonal hyperimmune serum and the other with monoclonal antibody. This treatment was carried out separately for each blot for 2 h at 37C with continuous agitation (40 rpm). The antibody treated blots were thoroughly washed in WB in three changes of 20 min each. This was followed by incubation of blots with anti-rabbit conjugate for the polyclonal hyperimmune serum treated blot and anti-mouse conjugate for the monoclonal antibody treated blot, for 1 h with gentle agitation (30 rpm). The unbound conjugate was removed by vigorous washing in WB, 4 times for 20 min. each.

The presence of adhered conjugate was assessed by freshly prepared and tested substrate. For this the NCP blots were dipped in substrate and agitated for 1-2 min.

To stop the reaction, NCP were washed in PBST/TGDW and bands visualized were recorded and photographed.

VIRAL DNA ISOLATION :

The partially purified virus suspended in TE buffer was used for DNA isolation following the method of Kennedy (1986) and Maniatis (1984). The whole procedure was carried out on ice and all reagents were refrigerated.

Materials and Reagents :

1. Phenol-chloroform : Distilled phenol was made 0.2% with 2-ME. The chloroform was a 24:1 (v/v) mixture of chloroform and isoamyl alcohol. The phenol-chloroform mixture was made 1:1 (v/v). This was kept in a dark bottle and TNE buffer was added to saturate the phenol.
2. Sodium acetate, pH 6.0 : A stock solution of 3 M sodium acetate was made in TGDW. The pH was adjusted to 6.0 with glacial acetic acid. This was dispensed into small vials and autoclaved at 15 lb pressure for 15 min.
3. Sodium dodecyl sulphate solution (SDS) : A solution of 10% SDS was made in distilled water. The solution was heated to 60C to dissolve the SDS and was stored in small vials at 4C.
4. RNase stock solution : A 20 mg/ml stock solution of RNase (Boehringer Mannheim) was made in sterile TGDW.

5. Proteinase K : A 10 mg/ml stock solution of proteinase K (Boehringer Mannheim) was made in sterile TGDW. This was dispensed in 300 ul to 500 ul aliquots and incubated at 37C for 30 min. to degrade any DNase.

Method :

The resuspended virus pellet was treated with RNase (final conc. 20 ug/ml) at 37C for 20 min. and then with Proteinase K (final conc. 500 ug/ml) at 37C for 20 min. This suspension was made 1.0 % with SDS (10%) and incubated over night at 37C.

The overnight suspension was transferred to 1.5 ml silicon coated sterile microfuge tubes. The sample tubes were washed with 250 ul of TNE which was added to the microfuge tubes. The maximum volume per microfuge tube was 0.6 ml. Equal volume of phenol-chloroform was added to this. The two phases were mixed thoroughly by gentle inversion for 1 min. This was then centrifuged at 12,000 rpm for 15 min. at 4C in microfuge (Remi high speed refrigerated microfuge). The upper extracted aquous layer was carefully removed into a fresh microfuge tube. The remaining phenol phase was re-extracted with 250 ul of TNE. Fresh autoclaved tips were used for pipetting. The aquous phase were pooled and the phenol-chloroform treatment was repeated once. The aquous layer thus obtained, was extracted with equal volume of chloroform-isoamyl alcohol. This extraction was also repeated once so as to remove all traces of phenol. The aquous phase obtained was made 0.3 M

in sodium acetate (3 M) and the DNA was precipitated by adding chilled absolute ethanol (AnalaR, Bombay) to a final concentration of 70%. The microfuge tubes were kept at -20C overnight. The DNA was pelleted at 10,000 rpm at 4C. The ethanol was decanted by inversion. The tubes were dried at 37C for 5-10 min. This pellet was dissolved in 100 ul of sterile TGDW and assessed for the presence of DNA.

DIGESTION OF VIRAL DNA :

The viral DNA was restricted with by three restriction endonuclease, (RE) enzymes viz., Eco RI, Bam HI and Hind III following the standard method of Maniatis (1984).

Materials and Reagents :

The RE enzymes along with their buffers (Boehringer Mannheim) were a generous gift from Dr. Donald Black, Pirbright, U.K.

Method :

The DNA solution of each isolate was divided into 4 microfuge tubes (One each for the three RE enzymes and one for control). The amount was made 45 ul with sterile TGDW to which 5 ul of stock enzymes buffer (10x) was added. To these were added 0.5 ul of RE enzymes. Sterile TGDW was added to the control. These tubes were incubated for 3 h at 37C. Thereafter, the mixture was run on agarose gel to assess digestion.

AGAROSE GEL ELECTROPHORESIS :

Standard procedure for agarose gel electrophoresis described by Maniatis et al. (1984) were used.

Materials and Reagents :

1. Tris-borate-EDTA (TBE), pH 8.3 : A 5x stock solution of 89 mM Tris, 89 mM boric acid and 3 mM EDTA was made in TGDW and kept at 4C.
2. Ethidium bromide solution : A 10 mg/ml stock solution of ethidium bromide (Sigma Chemicals, USA) was made in TGDW. This was stirred on a magnetic stirrer for several hours to dissolve the dye. Then it was dispensed in to vials, wrapped in aluminium foil and stored at 4C.
3. Agarose gel solution : Agarose (Pharmacia, Sweden) was made a 0.6% solution in 0.1x TBE. The solution was heated to boiling and then cooled to 50C.
4. Loading buffer : This buffer consisted of 0.25% bromophenol blue (Sigma Chemicals, USA), 0.25% xylene cyanol (Sigma Chemicals, USA) and 30% glycerol in TGDW. This was distributed in microfuge tubes and stored at 4C.

Method :

Place the casting tray with comb on a level surface. Pour into this the cooled (50C) agarose gel solution. The gel was allowed to set for 30 min. The comb was then removed and the gel placed in the electrophoresis unit so that the wells were towards the cathode (Black

cord). The cables were connected after loading (15 ul) the wells and a run at 60 V was made for 4 h.

The completion of run was assessed by the movement of the tracking dye whereafter, the gel was stained in ethidium bromide for 5 min. and visualized on a UV transilluminator. The bands visualized were photographed using 667 type film on a Polaroid camera fitted with an orange filter.

VIRUS ISOLATION :

The sample collected for virus isolation, as described earlier, were processed at the earliest. The media and reagents required were similar to those for cell culture.

Method :

1. Blood samples: The blood cells were centrifuged at 4C at 2,000 rpm. The WBC were collected and washed in MM. The cells were ruptured with autoclaved TGDW, centrifuged and the supernatant was used for inoculum (Singh et al., 1989) in MDBK cells.

2. Swabs : They were kept in TM containing antibiotic solution over night at 4C. Thereafter, the swabs were squeezed and centrifuged at 2,000 rpm for 20 min. The supernatant was filtered through millipore (0.45 u) and used for inoculating the MDBK cells.

3. Tissue materials : The tissues were triturated with autoclaved silica and MM. This was centrifuged at 2,000 rpm at

4C for 30 min. The MDBK cells were inoculated with the supernatant.

The inoculated MDBK bottles were incubated at 31C. Each of the inoculated samples were blindly passaged for 5 serial passages before discarding as negative. The bottles were observed for CPE upto 7 days PI. The samples were millipore filtered (0.45 u) after each passage. The samples which gave CPE for 5 serial passages were considered as virus isolate and were characterised.

VIRUS NEUTRALIZATION TEST, (VNT) :

To ascertain that the isolates exhibiting CPE were BHV-1, neutralization test was performed (House and Baker, 1971).

Materials and Reagents :

The materials and reagents required for VNT were similar to those required for titration of virus (described earlier).

Method :-

MDBK cell line was grown in 15 x 125 mm test tubes. The tubes with complete monolayers were used. A 10 fold serial dilution (10^{-1} to 10^{-10}) of the isolated virus was made in MM. Each dilution (0.5 ml) was incubated at 37C with 0.5 ml of standard antisera (constant dilution 1:50) for 1 h and at 4C overnight. Similar procedure was followed using the control standard negative serum (Flow Laboratories, USA). The test tubes were inoculated with 0.1 ml of each of the dilutions in a set of 3 tubes and incubated at 37C for 2 h. The inoculum was decanted and 1.5 ml MM was added to each of the tubes.

These were incubated at 31C. The test was recorded 72 h PI and titre calculated using the method of Reed and Muench (1938).

DOT IMMUNOBINDING ASSAY :

DIA was also done following the procedure already mentioned except that the dip-sticks were coated with 2 ul of this isolate (concentrated 10 times) and in the next step, the sera used were standard positive and negative BHV-1 serum.

IMMUNO-BLOTTING :

The new isolate was grown in bulk (Stock virus), pelleted, partially purified and run on SDS - PAGE in the similar manner as described earlier in this text. The transfer of proteins and further processing of NCP was also done following the described procedure, except that the proteins were transferred at 24 V for 3 h.

RESULTS

RESULTS

Bovine herpesvirus type-1 (BHV-1) infects a variety of domestic and wild animals to produce a long lasting humoral immune response. Therefore, the exposure of animals to infection in a particular geographical region could be demonstrated by detecting antibodies against BHV-1. The serological survey serves as an important epizootiological tool to study the dynamics of BHV-1 induced disease syndromes and detection of latent carriers in relation to disease control schemes and international trade.

Serological Survey by ELISA :

The extent of exposure to BHV-1 in cattle and buffalo herds in organized dairy farms of Haryana was assessed. Serum samples collected from exotic, crossbred and indigenous aborted and incontact cows and heifers, milch buffaloes and buffalo bulls of different organized livestock farms during 1987-88 and 1988-89 were tested for the presence of BHV-1 antibodies by ELISA.

The serological survey data obtained are summarized in Table 1. Of 48 indigenous cattle sera collected from GLF Sector 1, Hisar, 23 (47.91%) were found positive for BHV-1 antibodies. Twenty seven, 32, 64, 42 and 40 serum samples from crossbred cattle were collected, respectively, from GLF Sectors II and III, Hisar, Karnal, Ambala and HAU Animal Farm, Hisar, of which 17 (62.96%), 15(46.36%), 39(60.94%), 26(61.9%) and 17(42.5%) were positive for BHV-1 antibodies.

TABLE 1: DETECTION OF BHV-1 ANTIBODIES IN BOVINE SERUM SAMPLES (1987-88 and 1988-89)

Name of Farm	Exotic cattle			Cross-bred Cattle			Indigenous Cattle			Buffaloes		
	Total samples	Positive	Percent positive	Total samples	Positive	Percent positive	Total samples	Positive	Percent positive	Total samples	Positive	Percent positive
1.G.L.F.Sector 1,Hisar	-	-	-	-	-	-	48	23	47.91	-	-	-
2.G.L.F.Sector-II,Hisar	-	-	-	27	17	62.96	-	-	-	-	-	-
3.G.L.F.Sector-III,Hisar	-	-	-	32	15	46.36	-	-	-	-	-	-
4.NDRI,Karnal	-	-	-	64	39	50.94	-	-	-	-	-	-
5.Military Animal Farm,Ambala	-	-	-	42	26	61.90	-	-	-	-	-	-
6.H.A.U.Animal Farm,Hisar	-	-	-	40	17	42.50	-	-	-	-	-	-
7.CIRB,Hisar	-	-	-	*35	4	11.43	-	-	-	19	6	31.56
8.Indo Dairy hard Improvement Centre,Rohtak	-	-	-	-	-	-	-	-	-	30	13	43.33
9.Model Exotic Farm,Bhiwani	33	24	72.73	-	-	-	-	-	-	**10	4	40.00
Over all Total	33	24	72.73	205	114	55.61	48	23	47.91	49	19	38.78

Percent positive in cattle 50.16

Total Bovine Samples tested 380; Positive samples 188; Percent Positive 49.47

*Heifers excluded from total ** Buffalo bulls excluded from total

*Samples collected for 1987-88 and 1988-89 (Two years)

Thirty five heifers from HAU Animal Farm were also screened which revealed a mere 4 (11.43%) positive. Twenty four (72.73 %) out of 33 serum samples from exotic farm, Bhiwani, were positive for BHV-1 antibodies.

The presence of BHV-1 antibodies was also assayed in buffaloes. Thirty serum samples collected from CIRB, Hisar and 19 from HAU Animal Farm revealed presence of the antibodies in 13 (43.33%) and 6 (31.56%) of the animals, respectively. Of 10 samples collected from buffalo bulls used for semen export and semen banks, 4 (40%) were found to possess BHV-1 antibodies (Table 1).

A total of 380 bovine serum samples from indigenous, crossbred, exotic cattle and buffaloes were subjected to ELISA for detection of BHV-1 antibodies, of these 188 (49.47%) were positive. The overall percent positivity for BHV-1 antibodies was 50.16 in cattle farms and 38.78 buffalo farms.

The prevalence of BHV-1 antibodies in exotic cattle farm was 72.73% while in different crossbred farms it ranged from 42.5 % to 62.96% (average 55.61%) and in indigenous farm 47.91%. In buffaloes the prevalence of BHV-1 antibodies ranged from 31.56% to 43.33% (average 38.78%). It is apparent from the Table 1 that prevalence of BHV-1 antibodies was more in exotic cattle as compared to crossbred, indigenous cattle and buffaloes.

The comparative study on seropositivity of aborted and incontact animals for BHV-1 revealed that an over all of 73.4% of samples were positive from aborted animals while only 37.78% from incontact animals (Table 2 and Figure 1). Survey of exotic farm, Bhiwani revealed that 85.7% samples from aborted cases and 66.67% from incontact animals were positive for BHV-1 antibodies. Serum samples collected from crossbred aborted dams of GLF Sectors II and III, Hisar, HAU animal farm, Hisar; NDRI, Karnal and Military Farm, Ambala showed the prevalence of BHV-1 to the extent of 88.9%, 60%, 100%, 85.7% and 70%, respectively. The serum samples collected from incontact non-aborted dams of the same farms were, respectively, positive in 33.3%, 44.4%, 57.14%, 52.9% and 50% of the animals. The average seropositivity in aborted crossbred dams was 73.24% while in incontact it was 50% and in heifers 11.43%. Results obtained from indigenous farm showed a percent positivity of 55.5 in samples from aborted animals in contrast to 40 in incontact animal serum samples. Similar to this, the prevalence of BHV-1 antibodies in aborted buffaloes was 50% while incontact buffaloes it was 30%.

DIA as a Field Diagnostic Test :

The serum samples were further subjected to DIA to evaluate DIA as a field diagnostic test. The comparative results of both the tests (ELISA and DIA) are given in Table 3. It appears from Table 3 that both the tests have almost equal sensitivity. However, ELISA was able to detect

TABLE 2: COMPARATIVE RESULTS ON BHV-1 ANTIBODIES IN ABORTED AND INCONTACT BOVINE POPULATION (1988-89)

Name of the farm	Abortion cases			Incontact animals		
	Total samples	Positive by ELISA	Percent positive	Total samples	Positive by ELISA	Percent positive
^a Govt. Livestock Farm Sector-1, Hisar	9	5	55.5	20	8	40.0
^b Govt. Livestock Farm, Sector-II, Hisar	9	8	88.9	6	2	33.3
^b Govt. Livestock Farm, Sector-III, Hisar	15	9	60.0	9	4	44.4
^d Central Research Institute for Buffaloes, Hisar.	8	4	50.0	10	3	30.0
^b H.A.U. Animal Farm, Hisar	5	5	100.0	*42	8	19.1
^b National Dairy Research Institute, Karnal	14	12	85.7	34	18	52.90
^c Model Exotic Animal Farm, Bhiwani	14	12	85.7	6	4	66.67
^b Military Animal Farm, Ambala	20	14	70.0	8	4	50.00
Over all Total	94	60	73.40	135	51	37.78

Breed	abortion	incontact
a Indigenous	55.5%	40.0 %
b Cross bred	76.19 %	36.36 %
c Exotic	85.7%	66.7%
d Buffaloes	50.0%	30.0%

* Including 35 heifers with 4 positive

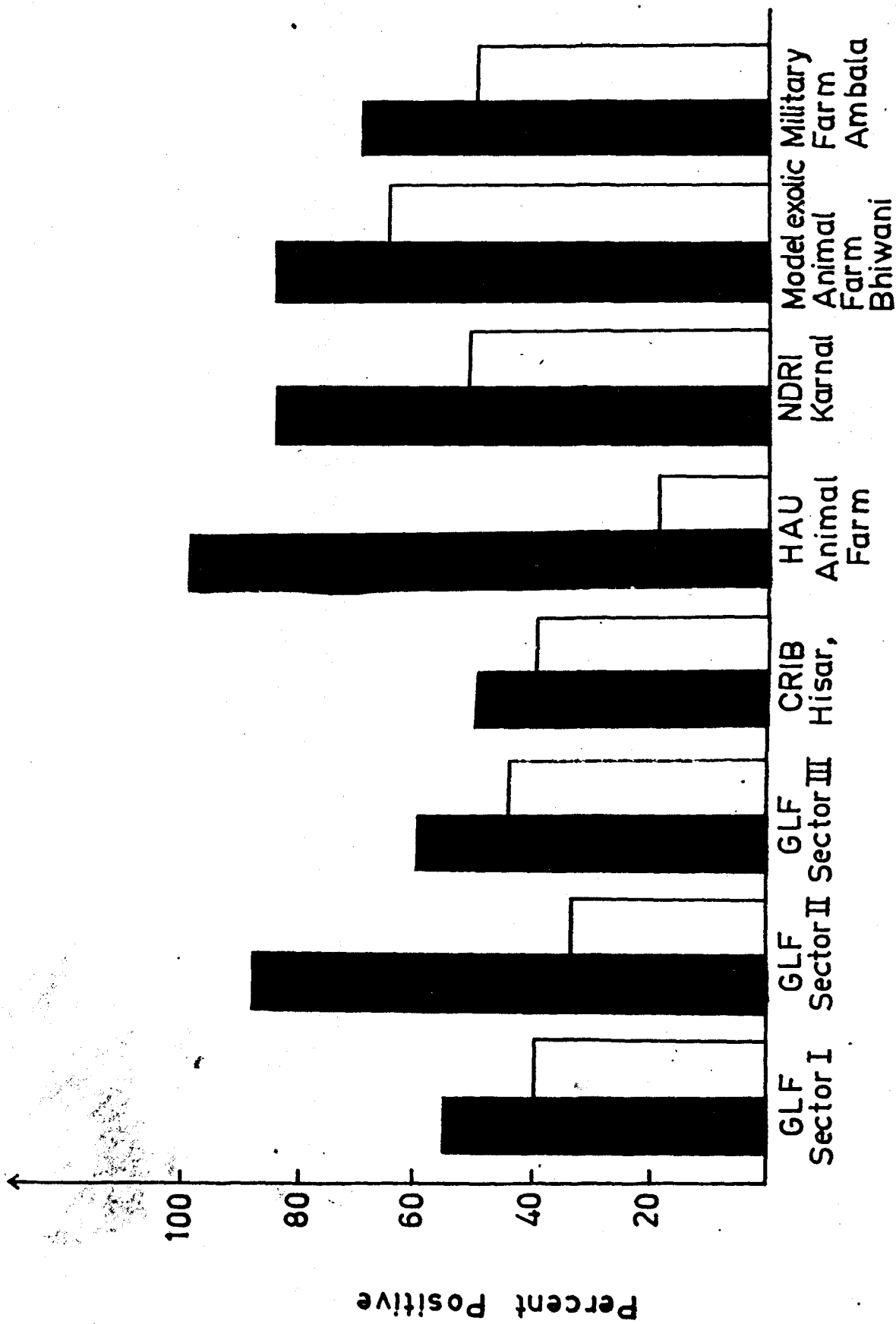


Figure 1. Percent Positivity to BHV-1 antibodies in aborted and incontact non-aborted animals in different Livestock Farms of Haryana (1988-89) ■ Abortion cases □ Incontact non-aborted animals

TABLE 3: COMPARATIVE RESULTS OF ELISA AND DIA FOR THE DETECTION OF BHV-1 ANTIBODIES IN BOVINE SERUM SAMPLES (1988-89)

Name of the farm	Total serum samples	E L I S A		D I A	
		Positive	Percent positive	Positive	Percent positive
Govt.Livestock Farm, Sector-I, Hisar	29	13	44.83	12	41.37
Govt.Livestock Farm, Sector-II, Hisar	15	10	66.67	10	66.67
Govt.Livestock Farm, Sector-III, Hisar	24	13	54.20	11	45.80
Central Institute for Research on Buffaloes, Hisar	18	7	38.89	7	38.89
H.A.U. Animal Farm, Hisar	47	13	27.66	13	27.66
National Dairy Research Institute, Karnal	48	30	62.50	27	56.25
Military Animal Farm, Ambala	28	18	64.28	16	57.14
Model Exotic Animal Farm, Bhiwani	20	16	80.0	16	80.00
Indo Dairyherd Improvement Centre, Rohtak	10	4	40.00	4	40.00
Over all Total	239	124	51.88	118	49.37

BHV-1 antibodies in 51.88 samples as compared to 49.37% by DIA. Furthermore, the samples positive by DIA were also positive by ELISA thus, indicating specificity of DIA.

Comparison of IVRI and NDDB isolates of BHV-1 :

Protein profile : The IVRI and NDDB isolates of BHV-1 were grown in bulk in MDBK cell line and were partially purified. They were then subjected to SDS-PAGE after a treatment with SDS. The results obtained are shown in Figure 2 and 3. As apparent from Figure 3, there were 26 bands in the IVRI isolate (lane 1) as compared to 25 in NDDB isolate (lane 2). The molecular weights of these polypeptides ranged from 14 to > 270 KDs. There were 4 bands which corresponded with the control cell line (68 KD, 48 KD, 36 KD and 34 KD). In the NDDB isolate 2 bands (molecular weight 140 KD and 33 KD) were not visualized and the VP10 had slightly different molecular weight (84 KD in IVRI and 82 KD in NDDB) while an extra band VP22 (molecular weight 25 KD) was present, as compared to the IVRI. The two isolates, except the above mentioned variations, show a similar protein profile.

Antigenic profile : The IVRI and NDDB isolates were further subjected to western blotting. The SDS-PAGE separated polypeptides were transferred to NCP and the antigenic profile of both isolates was studied using standard polyclonal hyperimmune serum against BHV-1. Both the isolates revealed 9 bands with the molecular weights ranging from 40 to > 220 KDs. The profile of both included

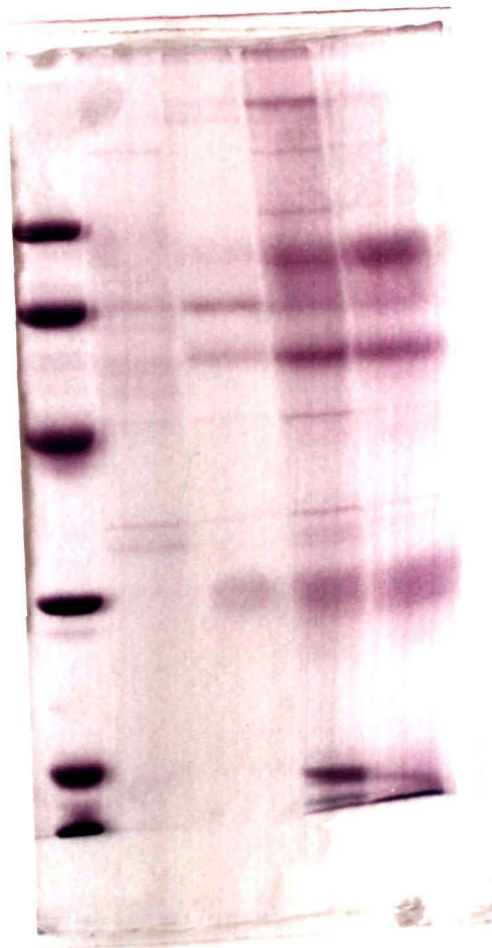


Figure 2. Protein profile of IVRI and NDDB isolates of BHV-1 by electrophoresis on 10% SDS-polyacrylamide gels stained with Coomassie blue.

- Lane 1. Standard molecular weight markers,
- Lane 2. Mock infected MDBK cells,
- Lane 3. Interphase of 40% sucrose cushion purification,
- Lane 4. IVRI isolate and Lane 5. NDDB isolate.

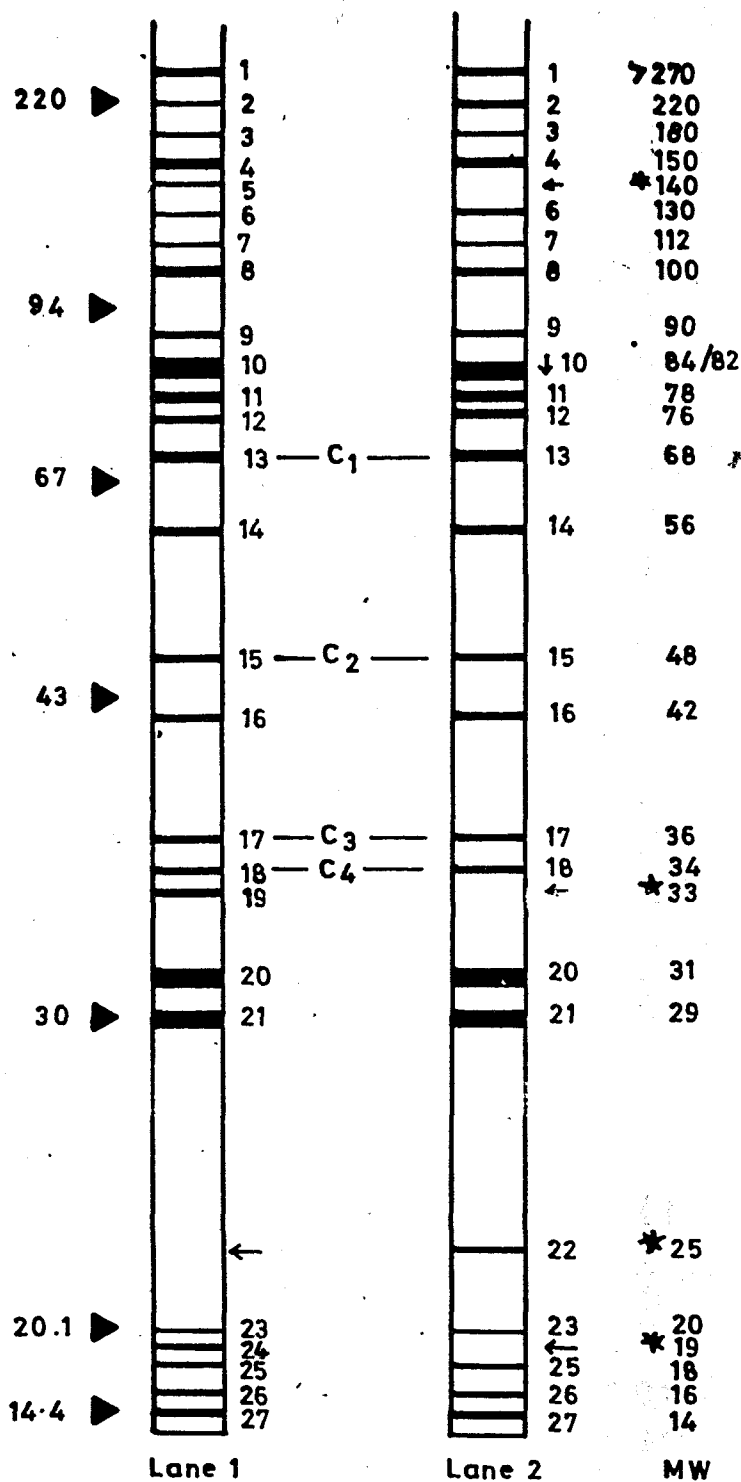


Figure 3. Protein Profile of the IVRI and NDDB isolates of BHV-1 using SDS-PAGE stained with Coomassie blue. Lane 1- IVRI isolate; Lane 2- NDDB isolate; C₁-C₄- bands in common with the control cell line.; ► Standard molecular weights in KD ★ bands missing in either of the lanes; MW calculated molecular weight in kilo dalton.

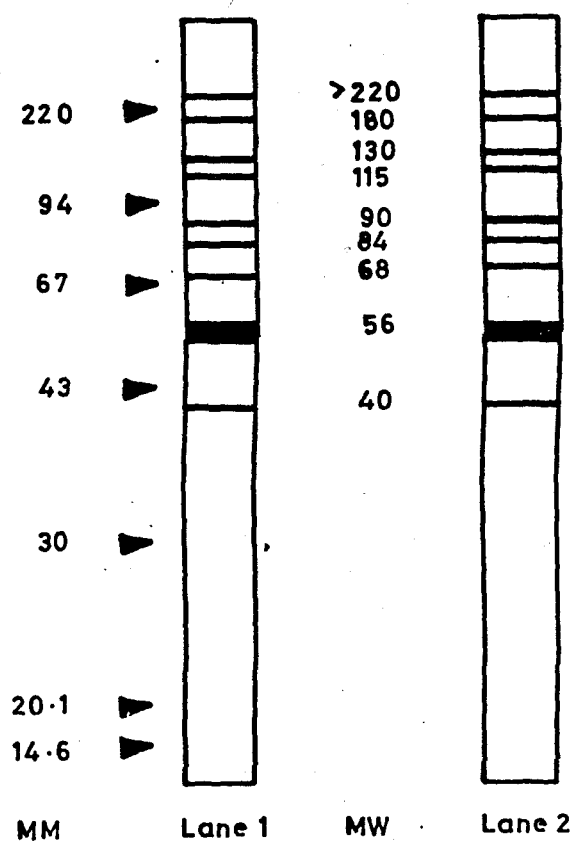


Figure 4. Antigenic Profile of Indian Isolates (IVRI and NDDB) of BHV-1 by western blotting using standard polyclonal antiserum. M.M.- Standard molecular wt markers; Lane 1- IVRI isolate; Lane 2 - NDDB isolate; MW: molecular weight in KD.

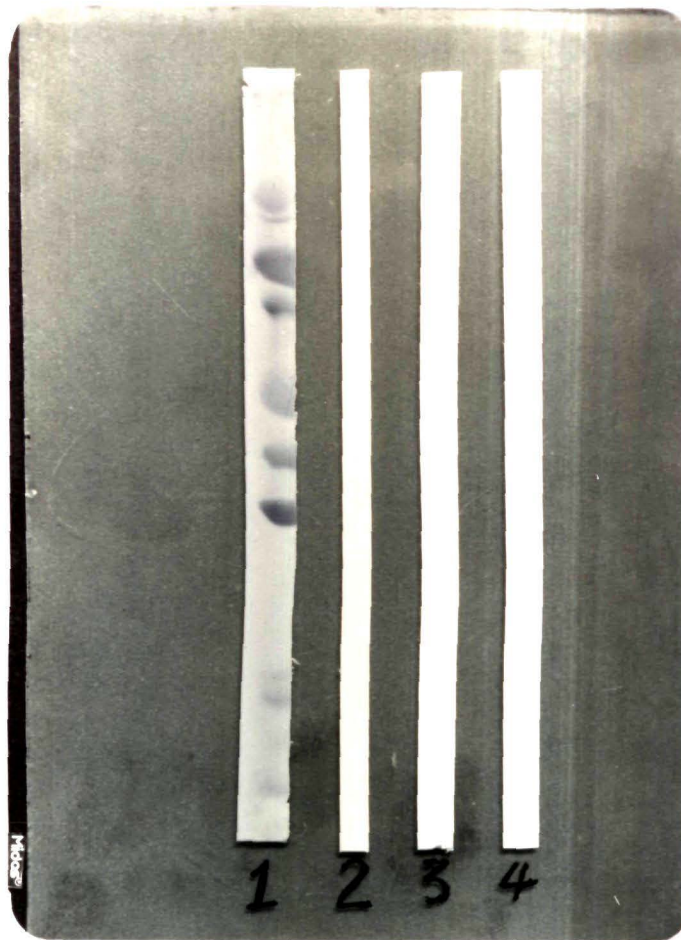


Figure 5. Western blot analysis of IVRI and NDDB isolates of BHV-1 as detected by monoclonal antibodies against the 90 KD hemagglutinin polypeptide.

- Lane 1. Standard molecular weight markers,
- Lane 2. Mock infected MDBK cell line,
- Lane 3. IVRI isolate and
- Lane 4. NDDB isolate.

the 90 KD hemagglutinin polypeptide. Thus, the profile of both the isolates was essentially the same (Figure 4). The presence of the 90 KD hemagglutinin polypeptide was confirmed by treating the NCP with the neutralizing monoclonal antibodies against an epitope on the hemagglutinin polypeptide. This gave a single band in the 90 KD region in both the isolates (Figure 5).

Genomic profile : A part of the purified pellet of both the isolates was used for DNA isolation. This DNA of both isolates was separately digested with Eco RI, Bam HI and Hind III and run on an agarose gel. On examining the ethidium bromide stained gel under UV transilluminator no band of the digested DNA was visualized while the undigested DNA band of high molecular weight was seen.

Isolation of BHV-1 :

Seventeen samples of blood, vaginal swab, liver, lung and nasal swab from 10 different animals which had history of abortion, (except animal No. F 986) were processed for isolation of the virus in MDBK cell line (Table 4).

Cytopathic effect (CPE) in MDBK cell line : Of 17 samples, 14 did not produce typical CPE till 5th passage and were considered negative for the presence of virus. Three samples of blood, lung and nasal swab from animal No. 206 J1.76 and foetus of A 461 exhibited CPE characterized by rounding, refractivity, detachment and protoplasmic strands connecting infected cells (Figure 6), from the 2nd passage to fifth serial passage. Of these the isolate from

TABLE 4: COLLECTION OF BIOLOGICAL SAMPLES FROM CLINICAL CASES FOR THE ISOLATION OF BHV-1 IN CELL CULTURE

Name of the Farm	Animal number	History	Samples processed	Cytopathic effect in MDBK cell line
H.A.U. Animal Farm, Hisar	91 ⁶¹	Abortion	Vaginal swab	-
	61 ⁴²	Abortion	Vaginal swab	-
	21 ⁵⁷	Abortion	Vaginal swab	-
Govt. Livestock Farm, Sector-II Hisar	J ₁ 111 _{3.74}	Abortion	Vaginal swab heparinated blood	- -
	J ₁ 206 _{4.79}	Abortion	Vaginal swab heparinated blood	- +
Govt. Livestock Farm, Sector-III Hisar	A550	Abortion	heparinated blood	-
	A551	Abortion	heparinted blood	-
			Vaginal swab	-
	A553	Abortion	Vaginal swab	-
			heparinated blood	-
	F986	Incontact non abortion	Vaginal swab	-
	A461	aborted foetus	heart blood liver lung nasal swab	- - NC* NC

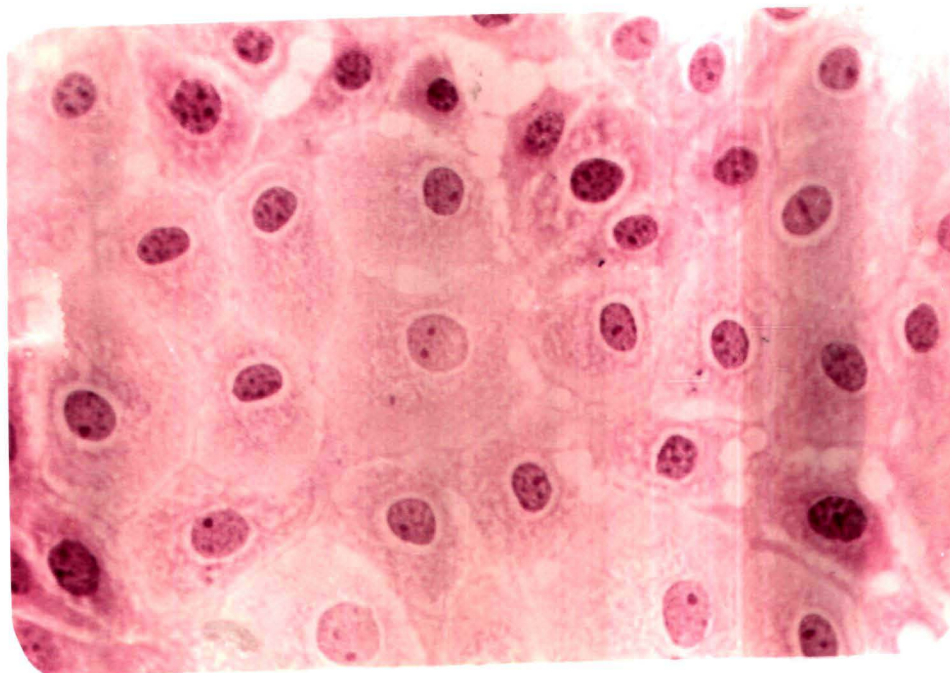
A Total of 17 samples were processed from 10 animals

*NC gave characteristic CPE upto 5 serial passages but was not further characterized.

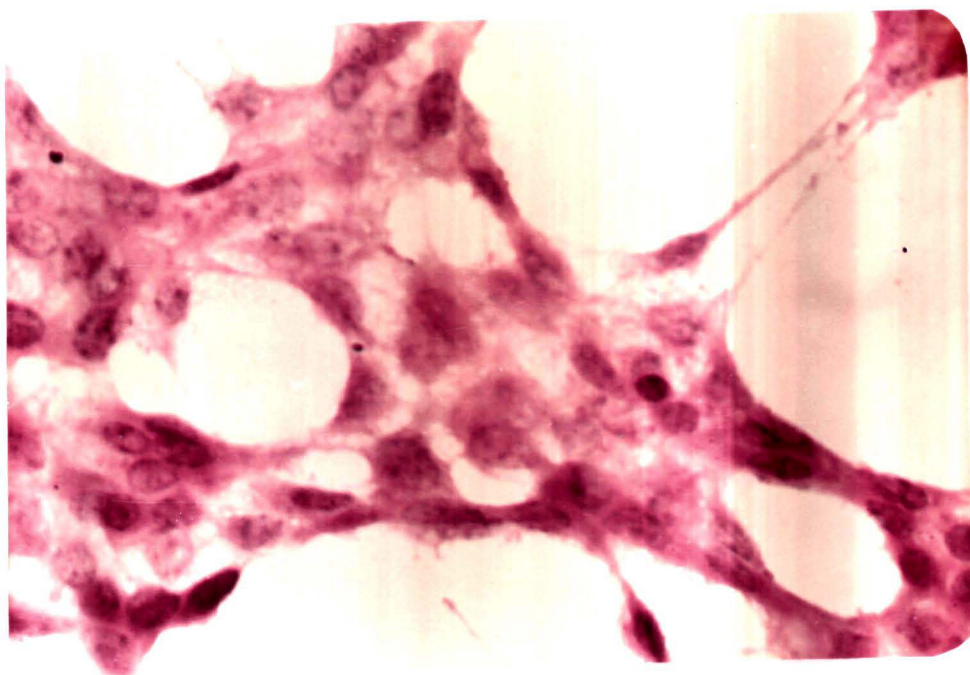
Figure 6. A. Mock infected MDBK cells 60 h post infected
(H & E stained)

B. BHV-1 infected MDEK cells 60 h post infected
showing rounding, clumping of cells and
protoplasmic strands joining the infected
cells (H & E stained).

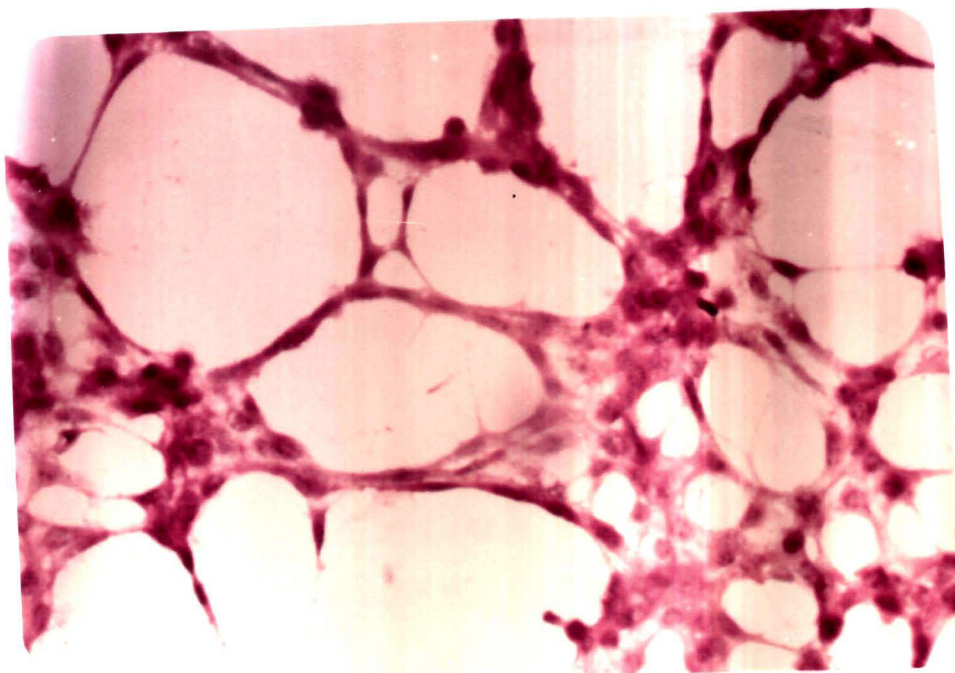
A



B



B



blood of aborted crossbred animal (206 J1.76) was further passaged in MDBK upto 7th passage and the CPE observed within 48-72 h was consistent in all subsequent passages. This virus isolate was further characterized.

Virus Neutralization Test (VNT) : Standard method of VNT for characterization of virus was employed in MDBK cell line culture using specific standard polyclonal hyperimmune serum against BHV-1. Specific neutralization was seen as indicated by reduction of the virus titre from $10^{6.5}$ TCID₅₀/ml to $10^{2.5}$ TCID₅₀/ml in presence of the standard antiserum. Thus, the virus neutralization index (VNI) was 4.

Dot Immunobinding Assay (DIA) : DIA using the new isolate as coated antigen and standard serum viz., polyclonal (Cambridge), monoclonal (Kansas University, USA) and standard positive antiserum (Flow Laboratories, USA), consistently gave positive results while with the standard negative serum (Flow Laboratory, USA), negative results were obtained.

Immunoblotting : The antigenic profile of the local isolate was analyzed by western blotting using the standard polyclonal BHV-1 antiserum. As shown in Figure 7 and 8, there were 11 bands with molecular weight ranging from 18 to > 270 KDs. Along with other polypeptides, the major 90 KD hemagglutinin polypeptide was also recognised by the standard polyclonal antiserum. These results further confirmed the presence of BHV-1 specific polypeptides in the local isolate.

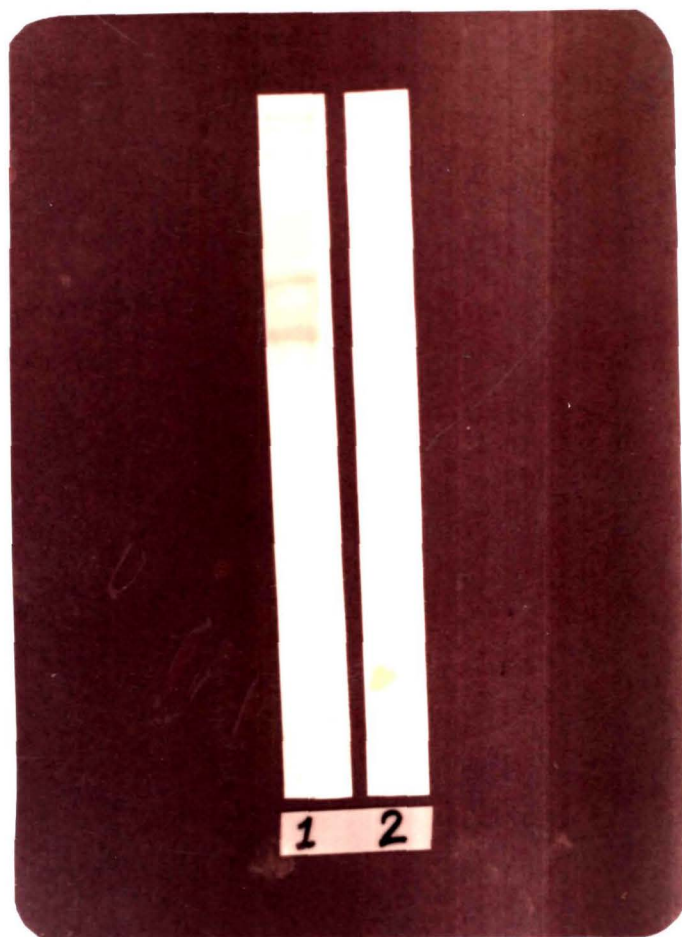


Figure 7. Immunoblot analysis of BHV-1 (Hisar isolate) proteins using standard polyclonal hyperimmune serum.

Lane 1. Mock infected MDBK cells and
Lane 2. Hisar isolate infected MDBK cells.

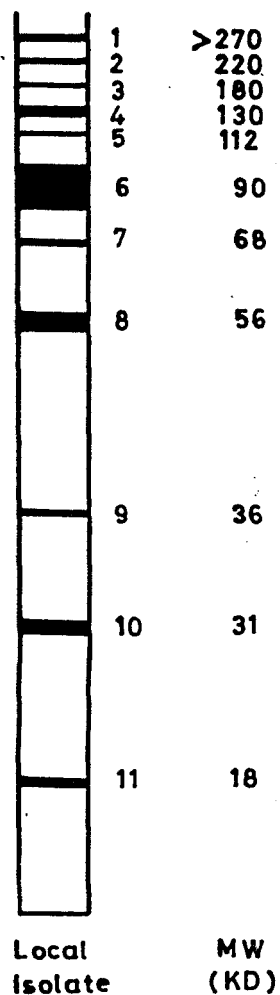


Figure 8. Antigenic Profile of Local BHV-1 isolate using standard polyclonal BHV-1 antiserum by western blotting.

DISCUSSION

DISCUSSION

BHV-1 is an infectious virus of bovine with numerous clinical manifestations leading to staggering economic losses by reduced productivity of the animals. BHV-1 has been mainly found associated with the ailments of respiratory, reproductive, ocular and nervous systems. Because of the complex interaction of this virus with different systems of the susceptible host, it has been very difficult to understand its pathogenesis at the molecular level. To evolve effective strategy for the control of this virus, research should be directed on the molecular understanding of the possible strains of BHV-1 in India and their association with the particular syndrome, if any. The other areas which warrant further investigations include epizootiology, sensitive and reliable diagnostic tools to understand the dynamics of BHV-1 induced syndromes in livestock. The present study is an attempt to study BHV-1 infection in the light of above discussed problems. Thus, the serological survey, isolation of BHV-1 from bovine in Haryana and differences in the protein and antigenic profiles, if any, of the BHV-1 isolates in India was conducted.

In the present serological survey, a single dilution ELISA was used for the detection of BHV-1 antibodies as described by Collins et al. (1985). The study revealed an overall percent positivity of 72.73 in exotic

breeds, 55.61 in crossbred, 47.91 in indigenous cattle, 11.43 in crossbred heifers, 38.78 in milch buffaloes and 40 in buffalo bulls. The overall positivity computed in bovine population from Table 1 showed 49.47 % indicating wide distribution of BHV-1 infection in the organized farms of Haryana. Similar to our observations, Mallick et al. (1986) reported 46.6% positivity rate in Haryana state by IHA and SNT. The slight difference in our overall positivity could possibly be due to use of ELISA in the present study, which is more sensitive than IHA and SNT. The prevalence of BHV-1 antibodies in India, reported by Mallick et al. (1986) was 73.26% in exotic, 63.46% in indigenous cattle and 64.24% in buffaloes which appear to be quite high as compared to our findings. This discrepancy could be because of the fact that in their study, samples were mostly collected from the animals showing BHV-1 associated syndromes. There are several other reports which indicated variation in the prevalence of BHV-1 antibodies in livestock population in India (Samal et al., 1981; Sulochana et al., 1982, Babu et al., 1984 and Singh et al., 1985).

The study of presence of BHV-1 antibodies in association with abortions showed 69 (73.4%) positive out of 94 serum samples screened from aborted bovine while only 51 (37.78%) were positive out of 135 samples screened from incontact non-aborting animals (Table 2 and Figure 1). The marked difference in the percent positivity between aborted and incontact animals indicated a positive association of

BHV-1 with abortions. However, it is difficult to suggest that all the BHV-1 antibodies positive abortions were due to BHV-1 alone, particularly when other incriminating agents causing abortions were not a part of the present investigation. Hence, the role of secondary invaders can not be ruled out. Moreover, BHV-1 has been reported to cause immunosuppression by activating suppressor T-cells (Splitter et al., 1986 and Miller-Edge and Splitter, 1986b). Therefore, BHV-1 infection may be a predisposing factor for infection by secondary abortogenic agents. Another mechanism by which BHV-1 could induce abortion indirectly is stress and loss of body weight due to syndromes of this virus other than reproductive.

The point prevalence of BHV-1 antibodies in aborted indigenous cattle was 55.5%, crossbred 73.24%, exotic 85.7% and buffaloes 50%. This indicated that the association of BHV-1 with abortions was more in the exotic animals as compared to the indigenous cattle and buffaloes. However, there seems to be no significant difference between the exotic and crossbred animals. Similarly, in the incontact non-aborting exotic animals, the point prevalence (66.67%) was higher than in other indigenous (40%), crossbred cattle (50%), and buffaloes (30%). Similar to these findings, Singh et al. (1985) reported 75% seropositivity in aborted animals and 44.4% in incontact animals of the northern region of India. Mehrotra and Rajya (1981) also reported 73% positivity rate in aborted animals.

Several other workers have reported sero-surveillance of BHV-1 in aborting bovine with percent positivity ranging from 23 to 57 (Mitchell and Greig, 1967, Samal et al., 1981, Babu et al., 1984 and Manickam and Mohan, 1987). The variation in the results of these workers could be due to different tests used, season, region, breed and age of animals included in the surveys.

As per the objectives laid down in the present investigation, dot immunobinding assay (DIA) was standardized and developed as a specific, sensitive, rapid and easy diagnostic test for field conditions. To establish the authenticity of this test 239 serum samples, randomly collected from different animal breeds of different organised farms of Haryana, were screened using DIA. The results were compared with the ELISA results of the same samples. The comparison of the two tests (Table 3) revealed no apparent difference in the positivity percent. Also there was not a single sample which was positive by DIA and not by ELISA indicating the specificity of DIA. Thus, DIA is as sensitive and specific as ELISA. Since DIA is on the whole easier to perform and more economical, requiring lesser amounts of reagents, it could be a better test for diagnosis of BHV-1 in field conditions. There seems to be no earlier reports on the comparison of DIA and ELISA for detecting BHV-1 antibodies in field sera, although, ELISA has been evaluated for screening antibodies against BHV-1 (Cho-II and Bohac, 1985, Collins et al., 1985, Edwards et al., 1986 and Riegel et al., 1987).

The two Indian isolates of BHV-1 viz., NDDB and IVRI, were compared on the basis of their protein and antigenic profiles. The protein profiles revealed minor difference (Figure 2 and 3). The partially purified IVRI isolate revealed a total of 26 bands as compared to 25 in the NDDB isolate. This included the four polypeptide bands (68, 48, 36 and 34 KDs) which were in common with the cell line control (MDBK). Trapaneir et al. (1986) reported 19 structural polypeptides, molecular weights from 14 to 147 KDs, in partially purified strains of BHV-1. The polypeptide bands visualized by them showed significant correlation with our findings. However, additional 10 polypeptides (> 270, 220, 180, 150, 78, 29, 25, 20, 19 and 16 KDs) were seen in our results. This could possibly be due to better migration and resolution of our gel system. In good accordance to our findings, Misra et al. (1981) reported 25-33 structural polypeptides related to BHV-1. Out of these, 11 were found to be associated with the virus envelope and were demonstrated to be glycoproteins (> 330, 182, 138, 130, 105, 82, 74, 55, 54, 49.1 and 42.2 KDs) which correspond to polypeptides 180, 140, 130, 100, 90, 76, 56, 48 and 42 KDs detected in the present study. Bolton et al. (1983) have also reported a total of 33 polypeptides with molecular weight ranging from 13 to 275 KD. This range coincided with our findings (14 to > 270 KDs) but the more number of bands in their study could be explained by their better resolution of gel with a slab gel scanner as compared to our visual resolutions.

The minor difference between the two Indian isolates of BHV-1 include the inability to visualize a 25 KD polypeptide in IVRI and 33 and 140 KDs polypeptide in NDDB isolate. Also VP10 of both isolates varied in weight (84 KD in IVRI and 82 in NDDB). Similarly, studies conducted earlier revealed no or minor differences in the protein profile of BHV-1 isolates (Pastoret et al., 1980, Misra et al., 1983 and Trepanier et al., 1986) but these were not conclusive enough to categorize the isolates into strains or associate them with a particular disease syndrome. The differences observed by these workers do not coincide with our observation. Also, differences by one of them do not coincide with the others observations. The differences in the molecular weights of the unvisualized bands of the two isolates in this study could not be made due to poor resolution which lead to inability to distinguish two overlapping co-migrating bands.

The two Indian isolates had also four bands in common with the control cellular profile (68, 48, 36 and 34 KDs). Previous workers (Trepanier et al., 1986) have also shown viral polypeptides in common with their control cell proteins. Moreover, these polypeptides which are in common with the cell control have been described as constituents of the virus (Misra et al., 1981 and Bolton et al., 1983). The bands in common with the control cell protein could be a part of the cell membrane which the virus acquired in its envelope at the time of budding from the host cell.

Marshall et al. (1986) have described a 64 KD protein of cellular origin associated with BHV-1 envelope. Trepanier et al. (1986) also reported a 45 KD cellular protein associated with BHV-1 structural protein. Furthermore, the 68 KD polypeptide of the virus which was in common with the cell control reacted with the polyclonal standard hyperimmune serum against BHV-1 in viral preparation but not in the cell line proteins. This indicated that the common 68 KD polypeptide was a part of the virus envelope which provokes antibody synthesis. The absence of reactivity with the cell control protein in the immunoblot could be because of the conformational or configurational changes of the common proteins in the virus envelope. The other possibility could be that 68 KD polypeptide band in cell control is an unrelated polypeptide having molecular weight similar to the viral polypeptides.

The western blot of both the Indian isolates revealed 9 bands using the polyclonal hyperimmune serum with no difference in their molecular weights (Figure 4). The 90 KD hemagglutinin protein which has a neutralizing epitope, was detected in both the isolates by monoclonal antibody against this neutralizing epitope (Figure 5). Similar to these results, Trepanier et al. (1986) reported the presence of this neutralizing epitope in the hemagglutinin protein of 14 isolates including the reference Cooper 1 strain. This major polypeptide was also detected by the polyclonal antiserum in both the isolates (IVRI and NDDB).

Trudel et al. (1986) reported nine polypeptides (105, 90, 74, 64, 53, 50, 46, 41 and 33 KDs) against which the convalescent serum reacted. Though in our study also, 9 polypeptides were recognised by the hyperimmune serum but only 4 (90, 68, 56 and 40 KDs) of these polypeptides corresponded with the findings of Trudel et al. (1986) (90, 64, 53 and 41 KDs). The 56 KD polypeptide band of the present study was a thick blurred band which might have included the 53 and 50 KDs polypeptides but this could not be confirmed. The remaining 5 bands (> 220, 180, 130, 115 and 84 KDs) did not correspond with the finding of Trudel et al. (1986). But the 115 KD, 90 KD and 84 KD proteins have been reported to have neutralizing epitopes by Collins et al. (1984) and Trepanier et al. (1986). The 180 KD polypeptide is reported to be the dimeric form of 90 KD monomer (Marshall et al., 1986 and Babuik et al., 1987) and 130 KD polypeptide a complex of 74 KD and 55 KD polypeptides (van Drunen Littel- van den Hurk et al., 1985). But as the exact weight of > 220 is not known it is difficult to correlate it with the known polypeptides. However, it could be the VP2 (263 KD) polypeptide mentioned by Misra et al., (1981) or the 240 KD by Scott et al., (1988).

The genomic analysis of the NDDB and IVRI isolates was attempted by digesting the viral DNA with Eco RI, Bam HI and Hind III. A single band of undigested viral genome control was detected while no bands were visualized in any of the lanes with restricted DNA. The inability to detect

bands in the RE digested DNA of NDDB and IVRI isolates could be due to less concentration of the viral DNA. Since these studies were inconclusive, further work on the genomic analysis is needed.

Seventeen samples from ten animals were processed for isolating BHV-1 in MDBK cell line (Table 4). Three samples gave CPE characterized by rounding, refractility and protoplasmic strands joining infected cells in five serial passage (Figure 6). Out of these three, one isolate from the blood of aborted crossbred cattle, was further characterized by VNT, DIA and immunoblotting. The drop in titre from $10^{6.5}$ TCID₅₀/ml to $10^{2.5}$ TCID₅₀/ml in presence of neutralizing BHV-1 standard antiserum confirmed that the isolate was BHV-1. Further, DIA using concentrated suspected BHV-1 isolate as antigen gave consistently positive results with both monoclonal and polyclonal standard positive antiserum. The new isolate subjected to immunoblotting using standard polyclonal BHV-1 antisera, revealed 11 bands (Figure 7 and 8). The results obtained by characteristic CPE, VNT, DIA and immunoblotting confirmed that the new viral isolate was BHV-1.

The animals from which the BHV-1 virus was not isolated does not imply absence of virus in those animals because isolation of the virus is not a straight forward task. It involves proper collection of sample at the proper time, its proper transportation, handling and processing. Also BHV-1 is known to become latent. Singh et al. (1986)

have also isolated BHV-1 in MDBK cell line from blood of aborted Holstein Freisien. The virus has also been isolated from systemic infections in calves, nasal swabs, conjunctival swabs, vaginal swabs and semen (Mehrotra et al., 1979, Singh et al., 1986 ,1987 and 1989 and Misra and Misra, 1987). The presence of BHV-1 antibodies and successful isolation of the virus from GLF sector II indicated that BHV-1 infection is prevalent in the farm.

The new isolate exhibited 11 bands ranging from 18 to > 270 KD in immunoblotting using polyclonal hyperimmune serum (Figure 7 and 8). Trudel et al. (1986) reported that there were only 9 polypeptides on the immunoblot using convalescent serum. However, the additional bands on immunoblot of the new BHV-1 isolate could be due to the use of hyperimmune serum in this study in contrast to convalescent serum used by Trudel et al. (1986). Besides the 7 polypeptides (180,130,112,90,68,56 and 36 KDs) in common to the IVRI and NDDB isolates of BHV-1 and discussed earlier , we detected 4 additional polypeptides (>270,220,31, and 18). Three polypeptides i.e. > 270 KD,31 KD and 18 KD correspond to the structural polypeptide VP1, VP6 and VP31 of Bolton et al. (1983) and VP2,VP24 and non structural (NSo) polypeptide of Misra et al. (1981). The possibility of the presence of antibodies against structural and non structural proteins in hyperimmune serum is quite strong. But the 220 KD polypeptide has not been reported earlier. However, it is near to the polypeptide 240 KD reported by Scott. et al. (1988).

The antigenic profile of the new Hisar isolate showed 7 bands in common with the IVRI and NDDDB isolates. There were two additional low molecular weight bands (31 and 18 KDa) and one high molecular weight band (220 KD). The 90 KD band of the new isolate was very thick and may have merged with 84 KD polypeptide seen in IVRI and NDDDB isolates. There was also a difference in the migration of the first polypeptide band (> 220 and > 270 KDa). Differing gel conditions are more likely to account for these discrepancies between molecular weights of the first polypeptide. Regarding the absence of two lower molecular weight (31 and 18 KDa) polypeptides, it is not possible to compare as the transfer conditions were different. The NDDDB and IVRI polypeptides were transferred over night at 4C at 12 V followed by 24 V transfer for 1 h just before finishing while the polypeptides of the new Hisar isolate were transferred at 24 V for 3 h at 4C. Thus, it is possible that the missing bands of lower molecular weights i.e., 31 and 18 KD in the over night run, being smaller thus faster migrating, might have passed through the NCP. However, to confirm whether the three Indian isolates of BHV-1 are similar, they should be subjected to electrophoresis under identical conditions (the same gel). This could not be done in the present study because of the technical difficulties.

The effective control of BHV-1 induced syndromes calls for, the development of a potent vaccine and sensitive diagnostic tools. To develop an effective potent vaccine detailed understanding of the genetic and antigenic profile of different virus strains circulating in the livestock population of India, needs to be done on priority. In the past couple of years, restriction pattern analysis and radioactive and non-radioactive DNA probes . are being increasingly and effectively used for identification of genomic differences and diagnosis of various viral infections of man and animals. Therefore, keeping in view, the modern trend in the strategy of understanding the molecular nature and pathogenesis of viruses, the techniques like restriction endonuclease analysis, DNA-DNA and RNA-RNA hybridization, hybridoma techniques and gene sequencing need to be exploited for study of BHV-1 and development of sensitive and reliable diagnostic tools and vaccine.

SUMMARY

SUMMARY

Bovine Herpesvirus type - 1 (BHV-1) is an important pathogen which inflicts heavy economic losses by various clinical syndromes in bovine. The virus is transmitted through semen, milk and fomites. Thus, BHV-1 is also a trade barrier for free international transport of animals and germ-plasm. In India, little work has been done to evaluate the extent of prevalence and the molecular nature of different BHV-1 strains circulating in livestock. The limited studies carried out hitherto indicated a wide spread prevalence of BHV-1 induced syndromes. Therefore, the present study was carried out with a view to determine the prevalence of BHV-1 antibodies in livestock population of Haryana, isolation of the virus and the strain variations in BHV-1 isolates.

In the present study, 380 serum samples from exotic, crossbred, indigenous and heifer cattle, milch buffaloes and buffalo bulls were collected from nine organized dairy farms viz., Government Livestock Farm, Sector I, II and III, Hisar; Central Institute for Research on Buffaloes, Hisar; HAU Animal Farm, Hisar; National Dairy Research Institute, Karnal; Model Exotic Animal Farm, Bhiwani; Military Animal Farm, Ambala and Indo Dairyherd Improvement Centre, Rohtak. The over all prevalence of BHV-1 antibodies by ELISA in Haryana state was 49.47%. The percent positivity was 72.73 in exotic cattle, 55.61 in

crossbred, 47.91 in indigenous, 11.43 in heifers, 38.78 in milch buffaloes and 40.0 in buffalo bulls. In aborted animals 73.4% serum samples were positive to BHV-1 antibodies while only 37.78% were in the incontact non-aborted samples. In the aborted animals, the positivity in exotic was 85.7%, crossbred 73.24%, indigenous 55.5% and buffaloes 50.0%. This indicated that the prevalence of BHV-1 antibodies was relatively higher in exotic and aborted animals.

Dot immunobinding assay was standardized and compared to ELISA to evaluate its sensitivity and suitability as a field test. A total of 239 serum samples were screened by both the tests. The results revealed 51.88 % positivity by ELISA and 49.37% by DIA. Thus, indicating that DIA was as good as ELISA. However, due to simplicity of method and economical considerations of DIA, it could be a better test for field diagnosis.

The protein profile of two Indian isolates (IVRI and NDDB) of BHV-1 was analysed on PAGE to compare the two. A total of 26 polypeptides in IVRI and 25 in NDDB were visualized (molecular weight ranging from 14 to > 270 KD). A polypeptide (25 KD) was not spotted in IVRI isolate and two polypeptides of 140 and 33 KDs in NDDB isolate. The molecular weight of VP10 was 84 KD in IVRI isolate while 82 KD in NDDB isolate. Included in the 25 and 26 polypeptides of the two isolates, four (68, 48, 36 and 34 KDs) were in common with the control MDBK cell line.

The antigenic profile of these two Indian isolates was studied by immunoblotting using polyclonal and monoclonal antiserum. The immunoblot with the polyclonal antiserum indicated presence of 9 polypeptides (> 220, 180, 130, 115, 90, 84, 68, 56 and 40 KDs) in both isolates with no difference in molecular weights. The monoclonal antibody against the 90 KD hemagglutinin protein was effective in detecting the corresponding 90 KD polypeptide in both the isolates.

Seventeen samples from 10 cows were processed for the isolation of BHV - 1. Of these 3 samples exhibited characteristic CPE in MDBK cell line from 2nd passage onward. Out of these, one was selected for further characterization. The virus neutralization test using standard polyclonal antiserum showed a neutralization index of 4.0 indicating that the suspected isolate was BHV-1. The isolate was further subjected to DIA and immunoblotting. Both these tests revealed positive specific reactions. The immunoblot revealed 11 bands (18 to > 270 KD). Therefore, on the basis of VNT, DIA and immunoblotting, the isolate was confirmed to be BHV-1.

The restriction endonuclease pattern of the two Indian isolates (NDDB and IVRI) was studied. The viral DNA was isolated and subjected to Eco RI, Bam HI and Hind III digestion. However, the results were inconsistent and further work is required.

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APPENDIX

Antibiotics stock solution :

Pencillin	10,00,000	IU
Streptomycin	2	g
Mycostatin	10,00,000	IU
HBSS	ad 100	ml

Seitz filtered and stored in aliquots of 10 ml at -20C.

Citrate buffer, pH 5.0 :

Solution A

Disodium hydrogen phosphate (anhydrous) 2.84 g/100 ml TGDW

Solution B

Citric acid 2.1 g/100 ml TGDW

Buffer

Solution A	25.7	ml
Solution B	24.3	ml
TGDW	50.0	ml

Electrode buffer (EB) pH 8.3 :

Tris	9.075	g
Glycine	43.2	g
SDS	3.0	g
TGDW	ad 3000	ml

The pH was adjusted with 1N HCl

Gel loading buffer :

Bromophenol blue	25	ul
Xylene cyanol	0.025	g
Glycerol	3.0	ml
TGDW	ad 10	ml

Stored at 4C in 10 ml vials after millipore filtration (0.22 u).

Phosphate buffer saline (PBS), pH 7.4 :

Sodium chloride	16.0	g
Potassium chloride	0.4	g
Disodium hydrogen phosphate (2 mol. water)	2.88	g
Potassium dihydrogen phosphate (, ,)	0.4	g
TGDW	ad 2000	ml

Running gel buffer :

Tris	22.714	g
TGDW ad	500	ml

Stored at 4C

Running gel solution :

Acrylamide	15.0	g
Bis acrylamide	0.4	g
SDS	0.15	g
Running gel buffer	ad 150	ml

Filtered through Whatman No. 1 and freshly prepared APS (10%) 375 ul and TEMED 37.5 ul were added just before use.

Sample buffer:

SDS	1.3	g
2-ME	2.5	ml
Glycerol	5.0	ml
Bromophenol blue (0.1%)	0.5	ml
Stacking buffer	25.0	ml
TGDW	ad 50.0	ml

Filtered before storing in small vials at 4C

Stacking gel buffer, pH 6.8 :

Tris	1.512	g
TGDW	ad 100	ml

The pH was adjusted with 1N HCl and stored at 4C.

Stacking gel solution :

Acrylamide	0.75	g
Bis acrylamide	0.02	g
SDS	0.025	g
Stacking gel buffer	25	ml

This was filtered through Whatman No. 1 and freshly prepared APD (10%) 60 ul and TEMED 6 ul were added just before use.

Transfer buffer (TB), pH 8.3:

Tris	9.075	g
glycine	43.2	g
Methanol	600	ml
TGDW	ad 3000	ml

Trypsin-phosphate-glucose-versine solution (TPGV), pH 7.2 :

Trypsin	4.0	g
Glucose	2.0	g
EDTA	8.0	g
PBS	ad 200	ml

Dissolved by stirring at 4C for 2 h with a magnetic stirrer and stored in sterilized 10 ml vials at -20C. This is diluted to working solution (1:20) in PBS.

Tris-borate EDTA buffer (TBE), pH 8.3 :

Tris	54	g
Boric acid	27.5	g
EDTA	5.2	g
TGDW	ad 1000	ml

This makes the 5x stock buffer which was diluted to 1x in TGDW before use.

Tris-EDTA buffer (TE), pH 7.4 :

Tris	0.121	g
EDTA	0.038	g
TGDW	ad 100	ml

This was adjusted with 1N HCl

Tris-sodium chloride-EDTA buffer (TNE), pH 7.6 :

Tris	0.242	g
Sodium Chloride	1.169	g
EDTA	0.075	g
TGDW	ad 200	ml

