STUDIES ON PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF BLUETONGUE VIRUS IN CELL CULTURE

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BY

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1999

Dedicated to

Maa Tarini

&

my beloved Parents

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Professor and Head,
Department of Bacteriology & Virology
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Dated. 25.08.1999

CERTIFICATE-I

This is to certify that the research work embodied in this thesis entitled **"STUDIES ON PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF BLUETONGUE VIRUS IN CELL CULTURE"** submitted by *Dr. Bira Kishore Parida* in partial fulfilment for the award of degree of **MASTER OF VETERINARY SCIENCE IN BACTERIOLOGY AND VIROLOGY** of the Orissa University of Agriculture and Technology, Bhubaneswar, is the original work, carried by the candidate himself.

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

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

25.8.99

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CERTIFICATE-II

Certify that the thesis entitled "**STUDIES ON PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF BLUETONGUE VIRUS IN CELL CULTURE**" submitted by *Dr. Bira Kishore Parida* to the Orissa University of Agriculture and Technology, Bhubaneswar, in partial fulfilment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** in the subject of **BACTERIOLOGY AND VIROLOGY** has been approved by the student's advisory committee. The candidate has carried out the original work sincerely and methodically.

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Bira Kishore Parida.
(BIRA KISHORE PARIDA)

ABBREVIATIONS

AGPT	Agar gel precipitation test
AR	Analytical Reagent
B-ELISA	Blocking ELISA
BTV	Bluetongue Virus
°C	Degree Celsius
CB	Cross bred
cDNA	Complementary DNA
C-ELISA	Competitive ELISA
CEF	Chick Embryo Fibroblast
CFT	Complement Fixation Test
CIE	Counter Immuno-Electrophoresis
CIFA	Central Institute of Freshwater Aquaculture
Cm	Centimetre
CPE	Cytopathic effect
DAB	Diaminobenzene
DCA	Deoxycholate
D-ELISA	Dot ELISA
DNA	Deoxyribonucleic acid
DPI	Days Post Infection
DPV	Days Post Infection
ds	Double Stranded
ECE	Embryonated Chicken Eggs
EDTA	Ethylene Diamino Tetra Acetic Acid
EHDY	Epizootic Haemorrhagic Disease Virus
ELISA	Enzyme Linked Immuno Sorbent Assay
FAT	Fluorescent Antibody Technique
FIG	Figure
FITC	Fluoresce in Isothio Cyanate
g	grams

GM	Growth Medium
GMEM	Glassgow modified essential medium
HA	Haemagglutination
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
H&E	Haematoxylin & eosin
HIS	Hyper immune sera
H_2O_2	Hydrogen peroxide
HRPO	Horse Radish Peroxidase
hrs	hours
H_2SO_4	Sulphuric Acid
I-ELISA	Indirect Enzyme Linked Immuno Sorbent Assay
IFT	Immuno Florescent Test
Ig	Immunoglobulin
IPT	Immuno Peroxidase Test
IV	International Unit
IUDR	5 Iodo-2-deoxyuridine
Kb	Kilobase
Kd	Kilodalton
LAH	Lact Albumin Hydrolysate
LT	Lamb Testicle
Mab	Monoclonal Antibody
MA	Milliampere
MDBK	Madin Darby Bovine Kidney
μg	Microgram
mgs	Milligrams
Ml	Millilitre
μl	Microlitre
MM	Maintenance Medium
mm	Millimetre
MOI	Multiplicity of Infection
NC	Nitrocellulose

nm	nanometre
OBPI	Orissa Biological Products Research Institute
OD	Optical Density
OIEA	Official International Des Epizootics Act
OPD	Orthophenylene Diamene
OPG	Oxalate Phenol Glycerine
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBP	Phosphate Buffer Saline
%	Percentage
PCR	Polymerase Chain Reaction
P.f.u.	Plaque Forming Unit
P.M.	Post Mortem
RBC	Red blood Cell
rDNA	Recombinant Deoxyribonucleic Acid
rpm	revolution per minute
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SNT	Serum neutralization Test
TCID50	Tissue Culture Infective Dose Fifty
TDW	Triple Distilled Water
TV	Trypsin Versene
u.v	ultra violet
V.P.	Viral Polypeptide
V.V.	Vaccinia Virus
V/V	Volume by Volume
WKS	Weeks
W/V	Weight by Volume
<	Less Than

CONTENTS

CHAPTER-I

INTRODUCTION

INTRODUCTION

Bluetongue (BT) is an important arthropod borne viral infection of domestic and wild ruminants which causes staggering economic losses to animal industry. The disease is widely prevalent in India and a number of serotypes have been reported from different parts of the country. The colossal economic losses due to BTV infection are mainly attributed to high morbidity, mortality, abortions, still births, foetal abnormalities, milk and meat loss. The presence of BTV imposes restriction of international trade on animals and their germplasms in exporting animals and their products to several countries. In India, there are highly desirable and outstanding breeds of cattle (*Bos indicus*) and buffaloes (*Bubalus bubalis*) and during recent years, several countries have shown interest of germplasm of Indian cattle and buffaloes for molecular and genetic study. The bluetongue disease have been reported in OIEA list which warrants strict quarantine measures and extensive testing of animals. This emphasises the importance of the disease in developing countries like India with vast domestic and wild ruminants. Since BTV is transmitted by culicoides midges, the vector presents another dimension to the occurrence of infection in different geo-climatic conditions.

Bluetongue disease was first reported in Africa by Hutcheon in 1881 under the name of "epizootic catarrh". The disease apparently remained confined to the African continent for nearly half a century.

probably due to lack of susceptible sentinel animals, insect vectors or movement of affected animals from Africa to other parts of the World. BTV infection in cattle was recognised as early as 1905 but more detailed description of bluetongue as a disease of cattle was reported by Bekker *et al.* (1934), prior to which BTV was supposed to be a disease of sheep only. In 1949, Gambles described a severe epizootic of bluetongue in cyprus. The disease was later recorded in Palestine and Syria (Kommarov and Gold smith, 1951). BTV appears to have spread to Pakistan through the countries of the middle east, as reported by Howell in 1959. It is logical to assume that BTV infection reached India via Pakistan either by movement of infected animals or due o insect vector since India has a very long boarder with Pakistan. However, the possibility of BTV having entered India through the importing of sheep and cattle from other countries, where BT is endemic, can not be ruled out.

The bluetongue disease was first reported by Sapre in 1964 in an outbreak of BT among sheep and goats in Maharastra. Bhambani and Singh in 1967 successfully isolated BTV from an outbreak in a Govt. farm at Utter Pradesh and reproduced the disease experimentally in sheep and later a number of workers reported the incidence of the disease from their respective state based on detection of antibody by AGPT.

India is now an endemic zone of BTV infection and outbreaks of BTV have been recorded from various parts of the country. Out of 24 serotypes of BTV isolated from different parts of the globe, so far 19 serotypes have been isolated in India. In India, there is no well defined

control strategy for BT as it exists in other countries. Vaccination is not performed against BTV due to non-availability of the vaccine. Moreover, the exact number of serotypes circulating in the country is still not known. Therefore , control of BT disease and prevention of spread of virus depends on sound and extensive understanding of epizootiology of BTV and suitable vector control programme.

The disease is caused by Bluetongue virus (BTV) which belongs to the family-Reoviridae, genus-Orbi virus (Borden *et al.*, 1971). Virus is 65-80 n.m in diameter and it possesses a double stranded (d.s) RNA genome (Bowne and Ritchie, 1970), comprising of 10 segments. The genome is enclosed by a capsid of complex nature containing 32 well defined protein capsomeres arranged in icosahedral symmetry. The icosahedral particle is surrounded by an easily removed outer capsid structure which lacks clearly visible morphological subunits. None of the viral protein is glycosylated. the capsid is composed of an inner core and an outer core and genome codes for 7 structural polypeptides. The inner core consists of 2 major components of VP_3 & VP_7 and few minor protein (VP_1VP_4 &VP_6) surrounding 10.d.S-RNA segment. The outer layer of capsid contains 2 major protein (VP_2 & VP_5). Both VP_3 & VP_7 codes for group specific antigen which can be detected by AGPT, CIE, CFT & ELISA(Della-porta *et al.*, 1983), where as VP_2 & VP_5 codes for serotype specific antigen which is detected by S.N.T and monoclonal antibody based competitive ELISA. Both VP_2 & VP_5 are major immuno-genic polypeptides and induce neutralising antibody while VP_3 & VP_7 include precipitating antibody.

The disease affects sheep of all ages and breed while cattle, goat and wild ruminants suffer much milder symptoms and may act as non-clinical carriers of the infection. The epidemiology of bluetongue depends on interactions of host, vector, climate and virus. It occurs most commonly in late summer, when the vectors, culicoides species are most numerous in the environment. After an extrinsic incubation period of 7-10 days, the virus is excreted in the saliva. It has been realised that the natural disease is transmitted by at least one of the 22 species of Culicoides in Africa and by *Culicoides variipenis* in the U.S.A. After entering the host, the virus replicates in hemopoietic cells and endothelial cells of the blood vessels. Adult sheep remains viremic for 14 to 28 days and in cattle, the virus can persist for as long as 10 weeks. In sheep the disease is characterised by fever which may last several days before hyperaemia, excess salivation and frothing at the mouth are noticed; a nasal discharge, initially serous but becoming mucopurulent and speckled with blood, is common. The tongue may become cyanosed, hence "bluetongue". There is marked loss of condition, and the sheep may die, often through aspiration pneumonia. The coronary bands of the feet exhibit hyperaemia and are painful. Oedema of the head and neck is not uncommon; animals with coronitis are often reluctant to walk and tend to be recumbent. Muscle degeneration occurs and in many animals, convalescence sense is protracted. Morbidity may be as high as 80 % and mortality 50 %. In contrast, the disease in cattle is usually in apparent and rarely acute. No clinical BT has been observed in cattle and buffaloes, but these animals may play an important role in the maintenance of virus in nature (Prasad *et al.*, 1992). Some stains of bluetongue may cause abortion, congenital abnormalities. In calves and

lambs infected in utero, viraemia may be present at birth and persist for several weeks (Frank and Fenner, 1993).

Orissa is an agricultural based state where 80 % of the population lives in rural areas. About 70 % of the rural population which include small, marginal and land less farmers, depend on agriculture and rear cattle, buffaloes, sheep and goats for their lively-hood. As per 1995-96 livestock census (Govt. of Orissa, A.R.D. Dept.) there are 14.76 million cattle, 1.65 million buffaloes, 1.86 million sheep and 5.41 million goats in the state. These animals are susceptible to various viral diseases amongst which bluetongue is one that causes serious economic losses to the animal industry in general and farming society in particular. The seroprevalence of the disease in the state has been reported by Behera (1997) to be 67 % in sheep and 28 % in goats and in cattle 61 % (Khan, 1998). It is therefore, necessary to conduct systematic studies on this virus for development of suitable immuno-prophylaxis against the disease.

Orissa is one eastern coastal state where climatic condition and ecology is most conducive to bluetongue. The state has got good monsoon period, long rainy season and abundant vector (culicoides) population which co-exist very closely with susceptible animal and the wild ruminants population and at any point of time the disease can flare up. Besides, as the disease is an economically important disease of domestic and wild ruminants and not much work has been done so far in Orissa and other adjoining states. on isolation characterization of virus and production of vaccine, it was envisaged to undertake studies on "Physico-chemical &

Biological characterization of Bluetongue virus in cell culture" which may have potential to generate very useful basic information on the properties of virus and provide suitable strategy for effective control of bluetongue disease in the state. The present study was carried out with the following objectives.

1. Adaptation and propagation of virus in cell culture.
2. Studies on cytopathic effect and growth kinetics.
3. Physico-chemical characterization of BTV such as size, nucleic acid type, sensitiveness to chloroform, formalin and other antiseptics, pH stability and thermal stability.
4. Study on seroprevalence of BTV in cattle by sandwich ELISA.

CHAPTER-II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

History

Hutcheon (1881) first gave a full description of a disease simulating BTV and named it as "epizootic catarrh".

Spreull (1902) suspected intra corpuscular parasite or plasmodium as causative agent of the disease and the name"Bluetongue" was coined by him (Spreull, 1905). He also reported that the virus was transmitted to goats, cattle and infection was in apparent.

BTV infection in cattle was recognised as early as 1905 but more detailed description of bluetongue as a disease of cattle was reported by Bekker *et al.* (1934).

Theiler (1906) established the filterable nature of the causal agent which passed through Berkefield filter and considered it to be a virus. It was closely associated with the blood but not exclusively with red blood corpuscles (RBCS).

Polson (1948) calculated a particle diameter of 100-150 mμ of BTV from ultra-centrifugation and ultra-filtration experiment.

Theiler (1908) reported that there was great difference in virulence of the various strains of BTV. He selected a mild strain of BTV

which was used for vaccine production after serial passages in sheep. The vaccine was used successfully for 40 years, although there were some reports of vaccine failures.

Alexander *et al.* (1947) serially passaged BTV in developing chick embryos for production of attenuated polyvalent vaccine.

Neitz (1948) clarified that inadequacy of vaccine against BTV was not due to poor immunogenicity of virus strain but because of the presence of plurality of strains.

Haig *et al.* (1956) successfully propagated BTV adopted the virus in tissue cultures which was found to be suitable for mass scale production of virus required for purification as well as for investigating various properties of virus.

In Asia the occurrence of BTV was first reported in Pakistan by Howell and Chow, (1969).

Sapre, (1963) was first to report on the prevalence of BTV among indigenous sheep and goats in Maharastra and in 1964 described the disease based on clinical pictures, post mortem lesions and AGPT.

Geographical distribution

From 1876 to 1940, BT was only reported in African continent (South Africa, 1976; Egypt, 1907; Kenya, 1909 and West Africa, 1927).

In 1943, the BT outbreaks were diagnosed in cyprus and up to 1949, it was reported from nearby areas of Asia, such as Palestine, Turkey and Syria. Subsequently it was observed that the disease showed its rapid spread through out the World like Eastern mediterranean countries (Gambles, 1949; Kommanov and Goldsmith, 1951); Northern America (Hardy & Price, 1952; Bowne *et al.*, 1964), Spain and Partugal (1956), the Ibrian Peninsula (Manso-Ribeiro *et al.*, 1957), Pakistan (1959) (Howell and Chow, 1969), Western Pakistan (Sarwar, 1962), Japan (Omori, 1961, Inaba *et al.*, 1966).

In India, Sapre (1964) was first to report an outbreak of BT in Maharastra and subsequently occurrence of B.T. outbreaks and its seroprevalence were reported from different parts of India. (Bhambani & Singh, 1968; Jooal & Vasudevon, 1980; Sodhi *et al.*, 1981, Lonkar *et al.*, 1983, Tongaokar *et al.*, 1983, Mehrotra and Shukla, 1984, Prasad *et al.*, 1987, Oberoi *et al.*, 1988, Mehrotra *et al.*, 1989, Mehrotra & Shukla, 1990).

In India, Jain *et al.* (1986) 1st isolated virus from sheep and in 1988 the same authors were successful in isolating the virus from Culicoides species.

PHYSICO-CHEMICAL PROPERTIES OF BTV

Physico-chemical characters of all the 24 different serotypes of B.T.V. were studied and experimental studies did not reveal any marked difference among them.

Nucleic acid type

Living Stone and Moore. (1962), Svehag. (1963), on the basis of staining of B.T.V. infected cells with acridine orange observed that, there were presence of clearly defined orange red inclusion bodies in the cytoplasm after 4 hours post infection. On the basis of this investigations, they concluded B.T.V. as a RNA virus. Bowne and Jochim (1967), described the presence of RNA positive intra cytoplasmic and DNA positive intranuclear inclusion bodies in BTV infected cells and thereby implying it to be a RNA virus.

Burke *et al.* (1968) studied the stability of virus to pH3 and IUDR (5-iodo-2 deoxy uridine) for nucleic acid determination, and indicated that the virus isolated, was RNA in nature.

Verwoerd (1969), (1970), have demonstrated that the genome of B.T.V consists of 10 segments of double stranded RNA like reovirus (Shatkin *et al.*, 1968) as its replication was not inhibited by IUDR, thus proving it to be a RNA virus.

Aruni *et al.* (1997) isolated and characterised B.T.V. to be a RNA virus as it was resistant to bromodeoxy-uridine (BODR) and Ribonuclease treatment.

Thermal Inactivation

Neitz (1948) reported that the stability of BT virus at ambient laboratory temperatures was attested to by the fact that it remained viable

in defibrinated blood for 25 years in South Africa. In the Denver Laboratory. infective blood has been routinely stored in OPG (an anticoagulant preservative solution consisting of potassium oxalate = 5g, phenol = 5 gm, Glycerin = 500 ml and distilled water = 500 ml) at 4°C for a year without any appreciable loss of titre and for several years with about a 50% loss in titre.

Neitz (1948) also recommended that the virus would not be frozen slowly at 10°C or -20° C because a severe loss on infectivity would result.

Svehag (1963) found that storage of virus for a month at 5°C or 22°C to 25°C, resulted in 50 % loss in infectivity, while at- 70°C or -27°C, no loss could be demonstrated. The author found that no infectivity remained after 3 years storage at 5°C or 22° C to 25°C and only 0.01 to 0,05 % of viral infectivity was left at -27°C. Even when stored at -70°C for 1 and 3 years, only 50 % and 5 % of infectivity, respectively were retained.

Wallis *et al.* (1962) reported that studied the thermostability of BTV by exposing the virus containing cell culture fluid at 56°C for 30 mts and found to be completely inactivated.

Svehag *et al.* (1966) reported that there was 100 fold reduction of infectivity titre of BTV after exposing to 56° for 30 mts. and retention of 10 % infectivity after 40 hours at 37°C (Svehag, 1963).

Foster (1969) described that undiluted embryo supernatant fluid infected with BTV was very stable at refrigerator (4°C) temperature

for over a year. He also observed that such fluid, partially purified by Genesolv-D treatment, lost infectivity rapidly when stored at 4°C.

Luedke (1969a,b) reported that it was necessary to store BTV infected blood in OPG at 4°C for 4 months to release virus so that it can be titrated via the yolk sac route of inoculation.

Mishra and Mehrotra (1996) described BTV to be heat labile. Aruni *et al.* (1997) also reported that the virus was stable at temperature at -196°C.

pH Stability

Wallis, Smith and Melnick, 1964 reported that Reo viruses were found to be stable between pH 4 & 8. BTV was slightly most stable at alkaline than of acid pH.

Owen (1964) reported that the persistence of B.T.V in carcass meat appeared to be dependant on P.M. changes and on the stage at which the animal was slaughtered. He also reported that the virus could survive for a period of 30 days at 4°C in mutton when pH did not fall below 6.3. No infective virus was isolated from an infected ovine carcass in which the pH of the meat dropped to 5.4. Bluetongue virus was isolated from the carcass of a bovine species 10 days after the animal had been artificially infected.

Svehag *et al.* (1966) and Inaba *et al.* (1970) studied sensitivity of BT virus to pH changes and found that virus was stable within narrow

range of pH 6 to 8 but inactivated below pH 6 and above pH 8 within a few minutes.

Jochim and Chow (1969) removed the virus from tissue culture fluid at pH 8.5, the pH maintained during 72 hour dialysis treatment and the precipitated infective virus retained a high degree of infectivity

Mishra *et al.* (1996) reported that the virus was readily inactivated at acidic pH 2.8 for 30 minutes.

Aruni *et al.* (1997) demonstrated that the virus is unstable in extreme alkaline pH but were stable at pH 6-8.

Effect of chemical agents

Mc Crory *et al.* (1959) evaluated the effectiveness of five chemicals such as Roccal (alkyldimethyl benzyl ammonium chloride), wescodyne (polyethoxy polypropoxy polyethoxy glycerol-iodine complex), sodium hydroxide, sodium carbonate and ethyl alcohol against bluetongue virus for disinfection purposes through tests involving the use of embryonating chicken eggs and sheep. In each of the tests the virus was used in chicken embryo material with 4 of the chemicals being inactivated for 4-6 minutes. Howover, Wescodyne at a final concentration of 750 ppm or greater was the only one that completely inactivated virus. The sheep serum was inactivated in a 5 mts inactivation period at 22°C by 750 parts per million of wescodyne, 3% NaOH, or 70 % ethyl alcohol in final concentration.

Howell (1963), Studdert (1965) Svehag *et al.* (1966). reported that BTV was resistant to ether.

Svhag, *et al.* (1966), and Bowne, (1965) reported at the same time that the BT virus was resistant to ether, chloroform and sodium deoxycholate but sensitive to treatment with trypsin. They also described that BTV was resistant to chloroform a solvent of even greater polarity than ether. He also reported that titres of the virus were increased 1.5 to 2.0 logs if the inoculum was treated with these two chemicals. It was reported that inactivation of BTV by trypsin was not by simply binding with viral receptors but by denaturation of viral particles.

Svehag *et al.* (1966) and Inaba *et al.* (1970) reported that BTV was stabilized with IM $Mgcl_2$ at 56° for 60 mts. Mishra *et al.* (1996) reported that the virus was found resistant or stable to chloroform, ether, sodium deoxycholate, sodium carbonate and was inactivated by 0.25 % trypsin as observed by Svehag *et al.* (1966). The virus was also inactivated by 3% NaOH, 70 % ethyl alcohol and 0.3 % formalin.

Aruni *et al.* (1997) stated that the BTV strains isolated from outbreaks were resistant to ether, chloroform and chymotrypsin and labile to trypsin treatment. They were resistant to bromoxy uridine and sensitive to ribonuclease treatment.

BIOLOGICAL PROPERTIES

In vitro studies

Propagation of virus in cell culture

BTV was found to multiply in number of cell cultures derived from different species and produce cytopathic effects. (CPE). Primary cultures of sheep kidney cells (Living Stone and Moore, 1962, Pini *et al.*, 1966. Girard *et al.*, 1967) cultures of BHK-21 clone -13 cells (Pini *et al.*, 1966) chick embryo cells (Inaba, 1975) were shown to support the growth of BTV. It was also reported that virus was susceptible to various cell cultures including primary foetal bovine and ovine kidney cells, African Green Monkey kidney (VERO) cells, Baby hamster kidney (BHK-21) cells, Mouse fibroblast (L) cells and foetal bovine bone marrow cells (Bando and jones, 1977a, 1977b, Stott *et al.*, 1978).

Fernandes (1959) described that 10 cell lines including Hela HB2 clone were susceptible to BTV.

Wechsler and McHolland (1988) reported the susceptibility of 14 cell lines to BTV infection.

Mishra *et al.* (1996) observed the response of BTV isolate in different cell lines and it was evident that the virus was adapted in BHK-21 clone-13, **VERO** and MDBK cell lines.

Cytopathic Effect (CPE)

Haig *et al.* (1956) developed a tissue culture technique and used it in SNT reported and successfully for the first time to adapt the virus in primary lamb kidney cells. Their brief description of the CPE caused by BT viral replication in the cells was confirmed.

Fernandes (1959a), was the first to directly grow B.T. virus from a blood of an infected sheep in tissue culture system. He studied the BT virus on 10 cell lines using phage-contrast microscopy, time lapse cinematography and routine histopathological staining. Fernandes (1959b) found that the giant cells produced by gamma irradiation of the cell culture were more susceptible to B.T.

Ohder *et al.* (1970) described that CPE produced in most of the cell culture systems and the changes were almost same. CPE was reported to be started usually after 40-70 hrs. but cellular destructions started as early as 24 hrs. In the beginning, CPE was confirmed to less number of cells and there was sloughing off cells. Afterwards it was found to spread rapidly and all the cells were affected (Howell and Verwoerd, 1971). Shrinkage of cells and increased granularity were the peculiar and specific cytopathic effect of BTV.

Mishra *et al.* (1996) observed that virus did not require any adaptation. In its first passage, CPE started coming by 24-48 hrs post infection. At this stage, cells showed rounding and there were formation of refractile cells with granular content. At a time, almost all the cells in the monolayer were affected. There were aggregation of cells and by 72 hrs. post infection, cells started sloughing off from glass surface.

Deshmukh and Gujar, (1999) propagated egg adopted 5-strains of BTV isolated from field sample in BHK_{21} upto 3rd passage and observed CPE like rounding, detachment, etc within a period of 96 to 120 hrs post infection.

Inclusion bodies

Bowne and Jochim, (1967) studied the ultracytopathologic response of cultured cells to BT virus replication. The first sign of abnormal activity is the formation of pinocytic vesicles in the plasma membrane. These vesicles were empty and reported to move to the interior of the cells where they cluster around the nucleus. Not all of the cytoplasmic vesicles originate from the plasma membrane as all membranous elements of the cell become vesiculated. Infact one type of inclusion body described by Bowne (1967) originated from the external nuclear membrane and contained a nonspecific granular matrix.

Bowne and Jochim (1967) reported that in later stages of maturation, there was extensive formation of vesicles in cytoplasm which was a prominent feature in BTV infected cells. In nucleus, granular masses with morphological structures appeared having similarity to nucleoli in the nucleus. These structures were reported as inclusion bodies type-1 which stained positive for DNA. Type-II inclusion (viroplasm) bodies were reported to be seen in the cytoplasm and stained positive for RNA.

Bowne and Jones (1966); Bown and Jochim (1967) and Lecatsas (1968) reported that ribosomes were often observed clustered around the periphery of this type of inclusion body and the present evidence indicated that the type-II inclusion body originates within a loop of rough endoplasmic reticulum. Another constant morphologic entity observed in conjunction with infected cells was large array of parallel tubular masses.

Bowne (1971) described that kinky filaments were clustered along with tubules and inclusion bodies in BTV infected cells, but there was no functional importance of these filaments.

Bowne and Ritchie (1970) observed that in the later stage, there was formation of extensive reticular structure in the cytoplasm after multiplication in viroplasm matured in those vesicles and released either by lysis or budding from the infected cells.

Brookes *et al.* (1993) described characterisation of virus and inclusion bodies in BTV infected cell. A combine qualitative and quantitative approach has been used to examine the role of virus inclusion bodies (VIBS) in the morphogenesis of BT virus and their number and profile areas increased significantly between 12 hr and 16 hr, and 20 and 28 hrs. P.I. respectively.

Brewer *et al.* (1994) observed that the replication of BTV in monocytes and stimulated lymphocytes were morphologically similar to that which occurred in vero cells with formation of viral inclusion bodies and virus specific tubules.

Mishra and Mehrotra (1996) biologically characterised BT virus by growing in vero cell line and observed acidophillic intracytoplasmic inclusion bodies at 48 hrs post infection, which were detected by haematoxylin and eosin stained coverslip preparation.

Growth curve

Mc Phee *et al.* (1982) conducted comparative studies on growth of Australian BT virus serotypes in continuous cell lines and embryonated chicken eggs. For comparative studies on replication rates, the 8 cell lines were infected within moments of each other with 0.2 ml inoculum, each containing the same amount of infectious virus. The no. of cell in monolayers varied with each cell line, however the MOI were estimated at 1 to 10 PFU per cell and 10^{-3} to 10^{-4} PFU per cell for a high and low MOI respectively. After 1 hr adsorption at 37°C, the inoculum was removed, the cells were washed once with PBS and replaced with 2.0 ml maintainance medium (for vero growth media=M199+5% FCS and for maintainance M199+3% FCS) and all the cell lines were incubated at 37°C. Duplicate samples were removed daily and immediately stored at -80°C until assayed.

Mishra *et al.* (1996) described the growth pattern of BT in various cell lines such as BHK21, VERO and MDBK. It was evident that multiplication of virus was rapid in BHK_{21} $clone_{-13}$ cell line as compared to other two VERO & MDBK. The virus was readily adapted to grow in BHK21 clone-13 cell line and the changes produced were similar to those reported by Bowne and Jochim, (1967).

SEROPREVALENCE OF BTV ABROAD

Song (1969) applied AGPT to detect seroprevalence of BTV infection in Korea and could record an incidence rate of 5.3%. of the disease among 225 cattle.

Hafez and Ozawa (1973) while carrying out seroprevalence of BTV observed an incidence rate of 8.9 % serum samples against 36.8 % in those history of bluetongue out break. In a similar attempt 20 % of buffaloes, 8 to 40 % of cattle, 17 to 39 % of goats and 14 % of camels were also positive for BTV precipitating antibody through AGPT (Egypt).

Afshar and Kayvanfar (1974) while examining 2921 serum samples obtained through slaughter house at Shiraz in Teheran, detected BTV precipitating antibody in 115 samples (7.6 %) from sheep 92 (13.6 %) from goats 41(0.6 %) from cattle 4(5.9 %) from camels and 1(4.5 %) from pigs through AGPT. However he failed to detect any positive precipitating antibody reaction from buffalo serum.

Carrier and Boulanger (1975) used soluble reactive preparation of BTV from infected suckling mouse brain through extraction and elution process in AGPT and could detect BTV precipitating antibody from 3 bovine serum samples of 13486 serum samples tested (Canada).

Guindo (1975) conducted an extensive serological survey against BTV with different animals through AGPT and found that 73 % camels, 10.7 % goats and 8 % cattle as positive reactors (Africa).

Hafez (1978) carried out serological survey in Iraq for BTV precipitating antibody from 3287 sheep serum samples of which 308 (16 %) developed clear precipitating lines while 421 (13 %) formed only spurs. In addition 42 of 178 goat serum (23 %) and 21 of 251 (9 %) cattle serum were found positive for BTV.

Pearson *et al.* (1979) used AGPT to screen BTV precipitating antibody from 5228 bovine serum samples and 447 sheep serum samples and could record 96% sample as positive through AGPT (U.S.A.)

Simpson (1979) conducted serological survey in Botswana for BTV precipitating antibody through AGPT and found 92 % cattle, 81 % camels, 83 % goats and 36% of sheep serum samples were positive.

Smith *et al.* (1981) conducted serological survey in Tennessee State of USA for a period lapsing from 1977 to 1979 with 429 serum samples from 6 to 9 month old calves and found 20 serum samples (4.6 %) were positive through AGPT for BTV.

Della-Porta *et al.* (1981) studied the seroepidemiology of BTV infection among northern Australia cattle and found that some cattle possessed BTV group reacting antibody and complement fixing antibody but not serum neutralising antibody to BTV 20.

Hayness *et al.* (1981) conducted an extensive serological survey for BTV precipitating antibody in cattle sera of Albama State in USA and found that 16% of the 1500 samples were positive through AGPT. North West and South West of Albama had approximately 22% and 27% of the samples were found positive compared to 6% and 8% for north east and south east Alabama. In addition altogether 70% of herds tested in Western half of Alabama state had antibody against BTV compared to 35% of the herd in the eastern half of the state. Thereby observing an overall BTV precipitating antibody positive herds to be 52 %.

Metcalf *et al.* (1981) observed BTV incidence rate 17.8% from 958 bovine serum samples by AGPT.

Liendo and Castro (1981) conducted an extensive seroprevalence study using CFT and AGPT for 3 yearsperiod ranging from 1st July 1978 to June 30th 1981 against BTV in Oklahona state of USA and found that while 1117 of 1629 (68%) of the Bovine serum samples positive by CF test, 49.5% (422 of 852 samples) as positive by both CFT and AGPT for BTV.

Fulton *et al.* (1982) carried out seroepidemiological study against BTV from 1978 to 1979 in Louisiana state of USA and found that 164 of 300 bovine serum samples (54.7%) were positive by AGPT. In the same study 58 of two herds (82.9%) and 164 of 597 (27.5%) individual cattle tested were found positive for BTV precipitating antibody.

Barzilai (1982) studied the seroincidence rate in Israel from camel sera and found that 56 serum samples when put to AGPT and micro SNT, 13 AGPT positive serum samples and 5 of 28 AGPT negative serum samples were found positive by micro SNT.

Abu Elzein (1984) while carrying out serological survey to BTV precipitating antibody from different animal from Sudan reported that 75% cattle, 80% sheep 14.6% dromedaries had the presence of BTV precipitating antibody through AGPT.

Hafez and Taylor (1985) in Saudi Arabia detected BTV precipitating antibody in 336 sheep serum samples of 560 (55%), 26 of 61 (42%) goats 18 of 112 (16%) cattle employing AGPT.

Homan *et al.* (1985) while conducting extensive serological survey for BTV precipitating antibody by AGPT found that 48.1% of 1435 cattle were positive. In a similar study from Columbia they could detect 51.8% of 635 cattle having precipitating antibody for BTV. In a significant note they could detect 0% and 19% cattle were positive for BTV precipitating antibody in Costa Rica and Columbia respectively at an altitude 2000 mts above while 61% and 67% cattle were found positive for BTV for low land cattle.

Pearson *et al.* (1985) employed AGPT for serological survey of BTV infection in cattle and found that 18.5% serum samples collected from 18 northern states from 1977 to 1984 were found positive.

Odiawa *et al.* (1985) while analysing the BTV and EHD incidence rate in Georgia state of USA found that 47% of 1068 cattle and 36% of 414 deer were having BTV precipitating antibody through AGPT.

Teclaw *et al.* (1985) in Mexican State of USA studied the serological incidence of BTV in 2156 cattle comprising 40 herds and observed that BTV prevalence rate varied from 7 to 100% with herd average of 42%.

Tamayo *et al.* (1985) from Chile, reported a BTV incidence rate of 19.6% of 1752 cattle in 99 herds while 65% individual herds had BTV precipitating antibody revealed through AGPT.

Stott *et al.* (1985) in an epidemiological survey on BTV incidence rate in California for 3.5 years observed that 41% of 8751 cattle 42% of 1469 sheep and 21% of 4785 goats had precipitating antibody against BTV as detected by AGPT.

Prasad *et al.* (1987) while analyzing an outbreak of BTV observed that 104 of 1177 serum samples from sheep (29,2%) and only 16 of 400 (4%) cattle sera revealed BTV precipitating antibody through AGPT. However, 60 indigenous cattle sera did not reveal any BTV precipitating antibody.

Shapiro *et al.* (1987) conducted a serological survey in cattle of British Columbia and South Western Alberta but could only record (0.1%) 5 of 4610 cattle sera to be positive through AGPT.

Afshar *et al.* (1989) while conducting serological survey of BTV incidence rate from dairy cattle, beef cattle, sheep and goat observed that 0.45%, 1.4% and 1.1% were found to be positive respectively by AGPT.

Fulton *et al.* (1989) while studying serological incidence of BTV in Mexico state of USA, observed that 16 of 40 Beef cattle (40%) revealed BTV precipitating antibody through AGPT.

Stott *et al.* (1989) investigated the serological evidence against BTV in Mexico state of USA from both cattle and sheep. While 9% of sheep serum sample revealed BTV precipitating antibody from 6 states, 35% cattle serum found positive for BTV from eleven states detected through AGPT.

Al-Busaidy and Mellor (1991) conducted an epidemiological study of BTV incidence rate in Oman comprising sentinel goat and cattle herds from 34 farms. They could observed a wide spread incidence rate of BTV in Oman.

Michel *et al.* (1994) investigated various factors like age, species, sex and location as regards to the seroprevalence of BTV in Australia and found that cattle over 4 years of age revealed higher percentage of BTV incidence rate (53.72%) compared to animals less than 2 yrs and 2 to 4 yrs of age group. Similarly, crossbred cases revealed 29.16% positive of BTV while *Bos indicus* species and *B. taurus* revealed 39.17% and 34.27% positive reactivity. Sexually intact males revealed highest percentage of BTV antibodies 60.56% against BTV while castrated male and female animals revealed 26.16% and 41.70% incidence rate respectively.

Ward *et al.* (1995a) while examining the field sera samples from Queensland, Australia against BTV through AGPT and SNT found that the estimated AGPT specificity was 69.5% with 94.7% sensitivity which was almost in par with the sensitivity and specificity of SNT.

Apiwatnakorn *et al.* (1995) conducted AGPT to detect BTV precipitating Antibody from 522 imported and domestic cattle, native sheep and goat in Thailand and found the incidence rate of BTV to be 28.6%, 39.4% and 73 respectively.

Ward and Carpenter (1995) used AGPT for seroprevalence study of BTV in Queensland of Australia comprising 410 herds of cattle and found that the mean herd prevalence rate was 5.2 % and median herd prevalence was 3.5 %.

Yunlong *et al.* (1995) conducted a seroepidemiological survey on incidence of BTV in Yunnan province of China comprising various animals like sheep, goat, dairy cows and Chinese yellow cattle, including 5 buffalos from vietnam and 23 yellow cattle from Myanmar. Total serum samples from these animals were 32821 of which 8253 (26%) revealed BTV precipitating antibody through AGPT. Buffalo and yellow cattle revealed the highest incidence rate of 34.1% and 30.6% respectively, while dairy cows revealed 0.8% BTV incidence.

Chang Gui *et al.* (1995) in a similar attempt screened cattle sheep and goat sera for presence of BTV precipitating antibody through AGPT and found an incidence rate of 1.5% and 2.2% for cattle and sheep and goat sera respectively.

Ward *et al.* (1995b) while carrying out serological survey for BTV precipitating antibody in Queensland state of Australia found that 8.7% of 20,000 cattle sera screened as positive by AGPT while 8.3% to 9.1% serum sample had neutralising antibody against BTV.

Lage *et al.* (1996) while applying AGPT and SNT to screen 329 serum samples could detect antibodies to BTV BHV-1 and BVD. While 179 serum samples from water buffalo of 329 (54.4 %) were found positive for BTV by AGPT, micro SNT revealed 34 of 238 (14.7 %) as positive for BHV-1 and 116 of 220 (52.7 %) as positive for BVD.

Counter immuno-electrophoresis (CIE)

Counter Immuno Electrophoresis was first used by Bussard (1959). later Colliford (1964) used CIE for determination of species origin from body fluid which later made it possible to detect both antigen and antibody.

Application of CIE was first reported by Gupta (1988) to detect BTV precipitating antigen. He used sonicated sheep blood cell for detection of BTV antigen and reported that CIE is more sensitive than AGPT.

Behera (1997) used extensively used CIE to detect BTV precipitating antibody and who observed that while AGPT could detect 39.19% for sheep and 10.60% for goat, CIE on the other hand could detect BTV antibody in 44.42% sheep and 24.02% of goat sera thereby detecting an increasing BTV sero-incidence rate of 5.33 and 13.42 for sheep and goat respectively.

Khan *et al.* (1998) screened of 499 cattle serum samples and observed that seroprevalence of B.T.V. was found to be 16.83 % through AGPT, 39.07 % by CIE and 67.33% by ELISA test.

Enzyme Linked Immuno Sorbent Assay (ELISA)

Engvall and Pearlman (1972) for the first time developed ELISA which has been frequently used now a days for detection of antigen and antibody because of its high sensitivity and specificity with various modification.

Hubschle *et al.* (1981) standardised and used indirect ELISA to detect BTV precipitating antibody from 10 experimental infected animal upto 63 days with various dilution. He could detect BTV antibody as early as 14 days reaching a peak at 42 days post infection. The O.D value for negative serum samples were 0.205, 0.177, 0.224, 0.198, 0.174 and the S.D. was 0.02184. Similarly, six positive control sera S.D. was found to be 0.260 and thus taken as cut off value and any test serum having O.D.value more than 0.26 was regarded as positive.

Poli *et al.* (1982) while attempting a comparative evaluation of ELISA, AGPT and neutralisation test with 126 serum samples which included 30 sero-negative samples of bovine sera infected experimentally found that 23% of the serum samples declared negative through AGPT were found positive by ELISA and similarly SNT being a type specific serological test could not detect the number of positive reactors declared by ELISA particularly when the serum samples were rich in IgG to group specific antigen.

Odiawa *et al.* (1985) conducted a survey of BTV and epizootic haemorrhagic disease (EHD) in ruminants of Georgia state of USA. The

frequency of precipitating antibody to BTV and EHD virus in 2200 cattle, sheep, goats and white tailed deer were 36% and 32% respectively.

Yang (1986) used structural polypeptide VP_7, which corresponded to the group specific antigen to coat the ELISA plates while testing 10% positive sera (1:400) and obtained the O.D. value varying from 0.322 to 0.795 (AV 0,62), where as using a whole purified virus antigen he could not get any difference. In their study the negative sera O.D. value varied from 0.182 to 0.221 (av. 0.195).

Afshar *et al.* (1987) used indirect ELISA and competitive ELISA to detect anti BTV antibody in sequential serum samples and elutes from whole blood dried on filter paper from 3 calves and 4 sheep experimentally infected with BTV-10. The Competitive ELISA was better for detecting anti BT antibody in the sera and whole blood samples from both cattle and sheep early after infection.

Drolet *et al.* (1988) used indirect ELISA and AGPT for bovine sera and antelope sera. They used a cell associated antigen for indirect ELISA and found that 14% of the cattle and 16% of the antelope had precipitating antibody against BTV which AGPT failed to detect, there by emphasising the sensitivity and specificity of indirect ELISA over AGPT.

Maggona *et al.* (1988) carried out a comparative study on seroprevalence of BTV infection from Oct. 1986 to Oct. 1987 in Lesbas and Rhodes with blocking ELISA and AGPT. Of the 65 sheep serum samples,

19 revealed positive or doubtful through AGPT, while 32 (50%) samples found positive through ELISA. Of the 71 bovine serum samples screened through AGPT, 41 were found positive while blocking ELISA could detect 58 (81%) samples as positive.

Anno (1988) Indonesia - in an annual serological survey conducted at Indonesia for BTV precipitating antibody through ELISA observed that 52% to 75% of large ruminants and 20% to 30% of small ruminants were positive to BTV.

Lunt *et al.* (1988) applied indirect ELISA with two types of antigens like a freeze thawed antigen and cytoskeletal antigen. He could note that cytoskeletal antigen increased the background which indicated that the freeze thawed antigen was superior to other antigen. The ELISA was able to demonstrate development and persistency of BTV antibody in cattle over the course of 120 days.

Afshar *et al.* (1989) compared competitive ELISA, Indirect ELISA and AGPT for screening BTV antibody from 1300 bovine sera 530 ovine and 160 caprine sera in bluetongue endemic 3 areas of Canada and 605 samples of bovine and ovine origin from USA and 79 cattle and sheep infected with 19 South African and 5 USA serotype BTV were subjected to both C-ELISA and indirect ELISA. The diagnostic specificity of competitive C-ELISA was superior and found to be 99.92% while that of indirect ELISA was 99.85%. At the same time specificity of AGPT was still less (99%).

Hugh *et al.* (1989) while carrying out seroprevalence study on BTV with 2550 cattle sera randomly selected from 274 louisiana herds from 1982 to 1984 employing ELISA, found that 39 % of the serum samples were positive.

Gupta *et al.* (1990) screened a number of sera sample by using dot ELISA and indirect ELISA and observed that dot ELISA to be a rapid and sensitive test.

Uhaa *et al.* (1990a) conducted a seroepidemiological study on BTV infection in dairy cattle in the cental valley of California to estimate the prevalence and distribution by age and season of BTV group reacting antibody. Between December 1985 and March 1987, samples of cattle ware tested at approximately 2 months interval for BTV group reactive antibodies using an ELISA. Of the 3774 serum samples tested, 238(6.3%) were from calves 1045 (27.6%) were from Heifers and 2492 (66%) were from cows. Seroprevalence varied carried from nil in calves on 2 occasions to over 90% on several occasions in cows. The seroprevalence of BTV group reactive antibody also showed a seasonal fluctuation with the highest rate occurring during the warmer months of the year. Relative estimate of sensitivity and specificity of BTV ELISA were 87% and 100% respectively compared to the standard AGPT. The indirect ELISA was more sensitive and specific than AGPT.

Uhaa *et al.* (1990b) evaluated the association between positive antibody response and production efficiency while screening antibody against BTV and *Mycoplasma bovis*. He found that 17.5% of 289 serum

samples had BTV precipitating antibody, 66.1% had antibody against *M. bovis* as detected by indirect ELISA.

Afshar *et al.* (1991) investigated the influence of gamma radiation on major group specific polypeptides of BTV VP_7 and found that higher exposure to radiation altered the reactive pattern of monoclonal antibody in ELISA.

Afshar *et al.* (1991) conducted a serological diagnosis of BTV in bovine and small ruminants by blocking ELISA or C-ELISA. Results from tests of blocking and competitive ELISA from 4 laboratories in Canada, USA, U.K. and Australia indicated that either of 2 tests could be adapted as an international test for the serological diagnosis of bluetongue virus infection.

Sendow and Daniels (1992) applied ELISA to detect BTV group infection. A competitive ELISA (C-ELISA) using a group specific monoclonal antibody (Mab) against the BTV was established to detect antibodies against BTV. Sera from sentinel cattle in West Java were tested. The results of C-ELISA were compared with the AGPT. Increase specificity was observed using the (Mab) in the BTV C-ELISA cross reaction to other orbiviruses was eliminated. It was concluded that the C-ELISA not only has increased sensitivity and specificity but also costless and can be used for all species and should replace the AGPT for the BTV seroprevalence study.

Afshar *et al.* (1992) analysed the efficacy of a blocking dot ELISA with that of C-ELISA from blood ellute samples from sentinel cattle

from Florida in USA for BTV antibody. Relative to C-ELISA results, the sensitivity of the B-dot ELISA was slight low (97%) and specificity was low (62.6%). Of the 635 bovine sera samples 12 and 84 resulted in false negative and false positive reaction for B-dot ELISA and C-ELISA respectively.

Afshar *et al.* (1992a) used indirect ELISA for simultaneous screening of bovine sera for detection of antibody to BTV and EHD with the help of monoclonal antibody (Mab) to bovine immuno-globulin (H chain) conjugated with HRPO. The performance of the combined indirect ELISA of course has many advantage over standard AGPT and it could detect separately either antibody to EHD or BTV.

Afshar *et al.* (1992b) used indirect ELISA for the detection of EHDV antibody with 3135 AGPT negative bovine field serum samples collected from Ontario, Alberta and British Columbia. Along with 130 AGPT positive samples the specificity and sensitivity of Indirect ELISA relative to AGPT were 99.3% and 91.5%. No cross reactivity was seen to EHDV antigen and BTV antigen in indirect ELISA.

Afshar *et al.* (1993) evaluated a commercial competition ELISA test kit for the detection of group specific antibody to BTV. The performance of a competitive ELISA test kit, blue plate special (BPS) for detection of group specific antibody to BTV was compared with that of an internationally endorsed C-ELISA. A total of 1026 serum samples of bovine and ovine-experimentally infected with different isolates of BTV serotypes from South

Africa-19, USA-5 and Australia-20 were tested. The sero conversion was found in all experimental animals inoculated with BTV with the exception of 4 cows in which there was a delay of 10-20 days. Similar to the ELISA-1 non of the sera from calves inoculated with USA and Australian Isolates of EHDV and Palyam virus cross reacted with BTV antigen in the (BPS) C-ELISA. The total agreement between the two assays for all the bovine and ovine field sera was 98.4%. The overall results substantiated the usefulness of (BPS) C-ELISA test kit for monitoring animal sera for group specific antibody to BTV.

White (1994) emphasized the usefulness of an ELISA based system to reliably assess the relative affinity of separate monoclonal antibody (Mabs) for heterologous isolates of BTV. He found that a BTV serogroup specific (Mab) possessed equivalent binding properties with the majority of the virus isolates tested.

Chang Gui *et al.* (1995) carried out an epidemiological survey on seroincidence of BTV in Jiangsu province of China where they could detect 68 of 3853 cattle sera (1.5 %) and 63 of 2426 (2.2 %) sheep and goat sera as positive through ELISA.

Zhou and Chan (1996) developed Western blot as a confirmatory test to the standard of competitive ELISA for sero diagnosis of BTV. Antimonoclonal antibody against VR_7 antibody and a polyclonal bovine BTV antiserum were used in blood analysis. PAGE separated partially purified VP_7 antigen was transferred to polyvinylidene difluoride (PVDF)

membrane. The immuno blot using HRPO conjugated antispecies immunoglobulin was used to screen 22 serum samples (7 from calves, 3 from sheep), alongwith C-ELISA and micro SNT. It is concluded that western blot is a sensitive, specific and rapid confirmatory test which can be used to detect group specific antibody against BTV.

Behera (1997) while carrying out BTV seroincidence rate by indirect ELISA observed that 165 of 292 serum samples (56.05%) were positive for BTV while 99 of 283 goat serum samples (34.98%) were positive for BTV.

Singer *et al.* (1997) employed C-ELISA to detect incidence of BTV with 26 cattle sera where the incidence varied from 17% to 89%.

Khan (1998) employed indirect ELISA for the detection of BTV antibody from 499 cattle serum samples and found an incidence rate of 67.33% in cattles of Orissa.

Seroprevalence in India

The earliest evidence of BTV infection in bovines in India is documented in the Annual Report of the Indian Veterinary Research Institute. According to this report, 3.7% of cattle sera were positive for BTV antibodies as detected by the agar gel immunodiffusion (AGID) test.

Bhambani and Singh (1968), Uppal and Vasudevon, (1980) reported the sero-incidence of BTV in other states based on the detection of antibodies by AGPT and SNT.

Sharma and Collegues (1981). Conducted a serological survey against BTV in cattle and buffalo in Punjab state. They recorded the presence of BTV antibodies in 6.8% of cattle sera. However, 40.6% of sahiwal cattle sera were positive, indicating the presence of BTV infection in indigenous cattle. In this study, more of the buffalo sera tested were positive for BTV antibodies.

Tangaonkar and Collegues (1983) in Gujarat state reported that 13.4% of buffalo and 15.6% cattle were positive for BTV antibodies through AGPT.

Bandyopadhyay and Mallick *et al.* (1983) recorded 3.7% of cattle sera to be positive for BTV antibodies by using AGPT.

Tangaonkar and Collegues (1983) tested sera from aborted and apparently healthy buffaloes in Gujarat and reported that 15.5% of samples was positive for BTV antibodies,. However, they could not correlate BTV infection with abortion. The positive sera revealed presence of antibodies against serotypes 1, 15 and 17.

Mehrotra and Shukla (1984) tested serum samples from 7 states (Andra Pradesh, Karnataka, Gujarat, Panjab, Orissa, Himachal Pradesh and West Bengal. A total of 154 cattle sera were tested through AGPT of which 28 (18%) were found positive for BTV antibodies.

Prasad and Colleagues (1987) failed to detect BTV antibodies in buffalo, while only 4% of cross-bred cattle sera were positive in the study conducted in Hissar of Haryana state. In this serosurvey, none of indigenous cattle sera were found to be positive for BTV antibodies.

Oberoi and Colleagues (1988) on a cattle farm in Ropar and a buffalo farm in Ludhiana, both in Punjab state demonstrated the presence of BTV antibodies in 37.5% of buffalo sera by AGID test. They also recorded 70 % of cattle sera to be positive for BTV antibodies.

Gupta and Colleagues (1990) conducted an extensive survey of BTV antibodies in cattle and buffalo in Haryana state. Serum samples were collected from a total of 549 cattle and 498 buffalo, of which 4.2% & 10.6% respectively were positive. In this study, 6.9% & 13.6% respectively of sera taken from males were also found to be positive.

Katoch and Sambyal *et al.* (1991) employed AGPT to detect BTV precipitating antibody from 258 serum samples collected from Gaddi sheep and goats, cattles and buffaloes from Himachal Pradesh. While he could not detect any BTV precipitating antibody with cattle sera or buffalo, a prevalence rate of 1.94 % and 0.83 % respectively could be found for Gaddi sheep and goat.

Naresh and Prasad (1995) used a C-ELISA directed against VP of BTV with 113 sheep 134 goats, 147 cattle and 43 buffalo serum for BTV precipitating antibody. Twenty five sheep serum (23.5%) from Haryana.

Himachal Pradesh and Punjab were found positive. Out of 134 goat serum samples screened from Haryana. H.P and Rajasthan 102 (67%) were found positive for BTV antibody. While of 137 cattle serum sample 83 (60.5%) were found positive for BTV, of the 33 buffalo serum samples collected from Punjab and Haryana, 26 (78.7%) were found positive for BTV antibody.

Behera (1997) extensively used CIE to detect BTV precipitating antibody and observed that while AGPT could detect 39.19% for sheep and 10.6% for goat, CIE on the other hand could detect 44.42% for sheep and 24.02% for goat thereby detecting an increasing BTV sero-incidence rate of 5.33 and 13.42 for sheep and goat respectively.

Khan (1998) screened a total of 499 cattle serum samples and observed the seroprevalence of B.T.V. to be 16.83% through AGPT, 39.07% by CIE and 67.33% by ELISA test. Thus, the over all seroprevalence of B.T.V. for cattle of Orissa was found to be 67.37%. ELISA could detect 28.26% and 50.5% more incidence rate of B.T.V. in cattle than CIE and AGPT respectively. The incidence of B.T.V. was recorded to be 56.75% for male calves and bulls where as in female calves and cows it was 70.36 %.

CHAPTER-III

MATERIALS & METHODS

MATERIALS AND METHODS

Bluetongue Virus (BTV)

BTV serotype-10 propagated in vero cell line was obtained through kind courtesy from Dr. S.K. Samal, Professor, Virology, Maryland School of Veterinary Science, U.S.A. and used in the study as a reference strain. This strain was further passaged 5 times in vero cell line and kept at 4°C for further use.

Cell lines

Vero cell line (African Green Monkey Kidney) was obtained from tissue culture unit, division of virology. Indian Research Veterinary Research Institute, Mukteswar, Kumaon, U.P.

Rabbits

Rabbits were obtained from Orissa State Biological Products Institute, Bhubaneswar.

Calf:

Six to seven month healthy crossbred calf, free from BTV antibody maintained at O.U.A.T. dairy farm, used for collection of blood (serum purpose for cell culture work). and raising hyper immune sera.

Propagation and titration of virus

Reference BTV serotype-10 was propagated in vero cell line using Glasgow ~~modified~~ minimum essential medium (GMEM). For this purpose, vero

cell was subcultured in milk dilution bottle having cell concentration $2x10^5$/ml. Following development of complete monolayer after 3 days of incubation at 37°C., the cells were ready for infection. Confluently grown vero cell monolayers were infected with a 10^{-2} dilution of stock reference BTV and allowed to adsorb for 1 hour and then washed 3 times with sterile maintenance medium (MM) to remove unbound virus particles. Cultures were incubated at 37°C to observe cytopathic effect for 5 to 7 days. After complete CPE, the bottles with monolayer cultures were subjected to 2 cycles of alternate freezing and thawing and were then harvested in sterile MC carteny's vial. The harvested virus was subjected to further 4 passage and distributed in small vials and stored at -4°C for further use.

The tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Muench with cells grown in micro-SNT plate.

PHYSICO-CHEMICAL PROPERTIES OF VIRUS

The physico-chemical properties of BTV were studied in vero cell line.

Thermostability

The method of Wallis *et al.* (1962), was used to study the thermostability of 5th passage level of reference BTV inoculum. For this, vero cells were grown in test tubes and infected with a 10^{-2} dilution of the stock 5th passage BTV and observed for development of CPE for 4-5 days. Following complete development of CPE, 3 sets of the infected test tubes were subjected to 2 cycles of alternate freezing and thawing, stored at -4°C.

The harvested virus was subjected to titration and for this, the inoculum was subjected to log dilution in maintenance media (GMEM). Vero cells were grown to confluent monolayers in 96 wells flat bottomed tissue culture Corning plate. Two rows of 6 wells were infected with log diluted inoculum ranging from 10^{-1} to 10^{-6} with 50μl/well in quantity. Following 1 hr absorption, the inoculum was decanted and wells were replaced with maintenance media. The plates were sealed properly and grown inside a desiccator with 5% CO_2 tension, suitable virus control and cell controls were also kept for comparison. Following 4-5 days of incubation at 37°C, the plates were observed for development of CPE and $TCID_{50}$ was calculated as per the method of Reed and Muench, (1938). To carry out the thermostability at varying temperature, one set of each harvested inoculum were subjected to temperature treatment at 45°, 50° and 56°C for 30 minutes in a water bath, and immediately transferred to an ice bath. All these 3 sets of tubes were subjected to titration in microtitre plate with vero cell grown in confluent monolayer, then the TCID50 was calculated.

Cationic stabilization

Virus cationic stabilisation was attempted according to the description of Yama Guchi *et al.* (1984). Virus material was subjected to 50° and 56°C for 1 hour in presence of IM magnesium chloride solution and immediately it was transferred to an ice bath. Finally the virus infectivity was determined by titration in vero cell line grown in microtitre plate.

Stability to pH

pH stability was studied by the method of Burke *et al.* (1968). Two sets of tubes each containing 0.2 ml of harvested inoculum, was treated with 2.8 ml of sterile M.M, while the other tube received 2.8 ml of citrate phosphate buffer to reach pH3 while another set of tube was treated with IM $NaHCO_3$ to bring the pH 8.2. Both the tubes were incubated at room temperature for 30 minutes and both sets were titrated in vero cell line to trace residual viability of the virus.

Chloroform sensitivity

Effect of lipid solvent like chloroform sensitivity was studied by the method of Feldman and Wang (1961) with slight modification. Harvested cell culture supernatant was treated with 10% v/v analytical grade chloroform and shaken for 10 minutes. It was further kept for 1 hr at 4°C. Following centrifugation at 1000 r.p.m. for 5 minutes, the aqueous low layer was carefully removed with the help of sterile pipette. Untreated infective fluid served as control and both were titrated in vero cell line. .

Formalin inactivation study

Infected cell culture fluid, was treated with 0.3% formalin analytical grade and the mixture was incubated at 37°C for 18 hrs. The residual infectivity was titrated in vero cell line. Untreated virus served as control.

Nucleic acid determination

For nucleic acid determination, the method of Burke *et al.* (1968) was used with slight modification. 5-iodo-2'-deoxy uridine (IUDR) (KOCH light, Laboratories ltd., Coln brok-Bucks, England) was used for the purpose. A stock solution of 1 mg/ml was prepared in double distilled water pH 7.4 and added to the maintenance media so as to reach final concentration of 50 μg of IUDR. Two sets of test tubes (15 in each set) with confluently grown vero cell monolayer were washed with sterile MM. About 1 ml of M.M. containing IUDR was poured to 1 set of test tubes while the other set served as control (without IUDR only M.M). Following 1 hour of incubation at 37°C, media from both the sets of test tubes were discarded. Stock virus inoculum was subjected to log dilution (10^{-1} to 10^{-6}) in sterile M.M. and 3 tissue culture tubes were infected with each dilution (0.1 ml/tube). After 1 hour adsorption at 37°C, the tubes were washed with M.M. The M.M. containing IUDR was added to each tube of the set previously treated with IUDR while the control tube received M.M. without IUDR. The tubes were further incubated at 37°C for 5 to 7 days and $TCID_{50}$ was calculated.

BIOLOGICAL PROPERTIES OF THE VIRUS

Infection of cell line

Vero cells were grown in confluent monolayer in milk dilution bottle with GMEM containing 10% calf serum (G.M.). The confluent monolayers were washed with M.M. and inoculated with 1.5ml of 10^{-3} dilution of 5th passaged BTV inoculum. For the detailed study of CPE produced by BTV, vero cells were also grown in Lighton's tubes containing

coverslip, and inoculated with 0.1 ml of viral inoculum. Following 1 hour. of adsorption to 37°C., the bottles as well as tubes were washed with M.M. and replaced with M.M. All the bottles and tubes were further incubated at 37°C for 5 to 7 days for the development of CPE. For the detection of inclusion bodies, Leighton's tube cultures were fixed with 10% formal saline solution after removing the M.M at 48 hrs and 72 hrs. The infected milk dilution bottles following complete development of CPE were freezed and thawed twice and kept at 4°C for further passage.

Fixed coverslips at various interval of incubation were stained with o.1% crystal violet and Giemsa' stain or haematoxylin eosin stain.

Growth curve study of BTV

For biological characterization of virus, growth curve study was attempted as per the method of Mc Phee *et al.* (1982). Briefly vero cells were grown in confluent monolayer in 35 test tubes. Thirty two test tubes comprising sixteen sets were infected with 5th passaged virus having titre of $TCID_{50}$ $10^{5.2}$(0.1ml/tube). After allowing 1hr. of adsorption at 37°C, the monolayers were washed with M.M. and replaced with 1.5 ml of M.M.. At 8 hrs. interval, one set of tubes were removed and stored at -20°C upto 96 hours till assayed. All these sets of tubes preserved were subjected to 2 cycles of freezing and thawing and the $TCID_{50}$ was determined according to the method of Reed and Muench with cell grown in 96 wells flat bottomed micro SNT plate.

ENZYME LINKED IMMUNO SORBENT ASSAY

A. Collection of serum samples

Blood samples were randomly collected from cattle of 12 organised dairy farms (livestock breeding farm) in the state of Orissa. Eight to ten ml of blood were collected in silicon coated vaccutainer tubes aseptically and the blood was allowed to clot. Serum was separated after centrifugation and kept in screw cap Mc Cartney's vials without any preservatives which were stored at -20°C till use. In the present study, a total of 220 cattle serum samples were collected from 12 government farm from different breeds, age and sex group of animals. (Table-1).

TABLE 1: COLLECTION OF SERUM SAMPLES FROM DIFFERENT ORGANISED GOVERNMENT LIVESTOCK BREEDING FARMS OF ORISSA

Sl.No.	Source of serum samples	No. of serum samples		Adult		Calves	
		CB	Exotic	Male	Female	Male	Female
1	Khapuria (Cuttack)	26	-	1	15	4	6
2	O.U.A.T (Bhubaneswar)	18	-	-	11	3	4
3	Remuna (Balasore)	21	-	2	13	1	5
4	Kathapal (Mayurbhanj)	8	-	1	5	0	2
5	Charbatia (Chaudwar)	12	-	-	9	1	2
6	Keonjhar	10	-	1	6	1	2
7	Chiplima (Sambalpur)	-	41	4	20	7	10
8	Sundurgarh	11	-	-	11	-	-
9	Bhanjanagar (Ganjam)	17	-	1	12	1	3
10	Bolangir	9	-	-	6	1	2
11	Phulbani	11	-	-	11	-	-
12	Sunabeda (Koraput)	-	36	4	32	-	-
	G. Total	143	77	14	151	19	36

B. Preparation of anti-BTV serum in rabbit

Ultrapurified BTV was mixed with an equal amount of Freund's complete adjuvant (FCA) and was used to immunise two rabbits as per the immunization schedule (Ey., P.L., Immuno-chemistry, 1978). The rabbits were inoculated with 1ml of inoculum each, intramuscularly (0.5 ml in each thigh) of 7 days interval for 3 injections and the last one was given I/vly for boosting the antibody production. After 10 days of last injection, test bleeding was carried out. The separated serum was subjected to AGPT & CIE & following the development of precipitating band, final reading was carried out, the serum was separated and stored at -20°C in a screw cap vial.

C. Preparation of reference bovine BTV antibody

Immunization of calf with ultra purified antigen was carried out to raise reference positive bovine BTV antisera. Prior to the immunization, blood sample was collected and serum served as BTV negative serum control. The immunization was carried out as already mentioned for the immunization of rabbits.

D. ELISA antigen

BTV serotype-10 ultrapurified antigen was obtained from Dr. S.K. Samal, Professor, Maryland School of Veterinary Science, U.S.A.

E. Sandwich ELISA

A poly clonal base double antibody sandwich ELISA was used in the present study to screen the cattle sera for the presence of BTV antibody. Ultrapurified reference serotype 10 was used as an antigen.

Standardization of ELISA

Sandwich ELISA was performed as per the methodology laid down by Western Brink *et al.* (1985) with slight modification. To start with two columns of Nunc modules were coated with 50μl of varying dilution of rabbit antibody against BTV like 1:50 to 1: 3200 (Two fold serial dilution) and kept at 4°C overnight. The following day the plates were emptied and washed three times with PBS twin-20 (pH 7.4) and the wells were blocked with 3% LAH (50μl) and incubated at 37°C for 1 hr. After blocking, again plates washed thrice with washing buffer and reacted with 50 μl of BTV antigen at a fixed dilution of 1:500. The plates were further incubated at 37°C for 1 hr following which washed thrice with PBS twin-20 and reacted with a fixed dilution of reference positive BTV antibody (50 μl) of 1:500 dilution with additional incubation of 1 hr at 37°C. The wells were washed thrice with PBS tween-20 and reacted with 50 μl quantity of antibovine HRPO labelled conjugate (Sigma) of 1: 5000 dilution. Incubation was carried out at 37°C for 1 hour after which wells were washed with washing buffer and reacted with 50μl quantity of substrate solution ABTS (Kirkegaard and Perry laboratories, USA). After 2-5 minutes when chromogenic reactions become evident, reaction was stopped by adding 50 μl of 5% S.D.S. and O.D. was recorded at 410 nm. in Multiskan, ELISA reader (Flow laboratory) and The O.D. value exceeding 0.400 and above was taken as cut off value as compared to O.D. value of negative rabbit serum and the corresponding final dilution of coating antibody was fixed at 1:400.

Titration of antigen

Following the determination of coating antibody dilution, the wells were coated with 1:400 dilution of coating antibody in 50 μl quantity per well in two rows of Nunc modules with coating buffer, and kept at overnight incubation at 4°C. Following overnight incubation, the wells were washed with PBS tween-20 and reacted with 50μl quantity of varying dilution of ultrapurified BTV antigen ranging from 1.100 to 1:12800 and similarly a negative antigen control i.e. normal vero cell lysate was subjected to same dilution and reacted with coating antibody. Rest of the procedures remained same as mentioned above and O.D. value exceeding 0.200 was taken as cut off value for the final dilution of antigen in comparison to negative antigen control and the corresponding dilution was fixed at 1:3200 for final dilution of the antigen.

Titration of test antibody

For the double antibody sandwich ELISA to carry out with the test serum samples, the reference positive calf BTV serum and the BTV negative were subjected to varying dilution ranging from 1: 100 to 1: 3200. Initially, the two rows of Nunc modules were coated with BTV rabbit antibody at a dilution of 1:400 in 50μl quantity. Following overnight incubation at 4°C, the wells were washed with PBS tween-20, blocked with 50 μl of 3% LAH and further incubated at 37°C for 1 hr. The wells were washed with PBS tween-20 and reacted with 50 μl quantity of 1:3200 dilution of BTV antigen and incubated at 37°C for 1 hr. After incubation the wells were washed with PBS tween-20 and reacted with above mentioned

dilution of reference positive sera in 50 µl quantity in one row of modules while the second row received the negative serum dilution. Following 1 hour of incubation at 37°C the wells were washed thrice with PBS tween-20 and reacted with conjugate and substrate as already mentioned and O.D. value of 0.088 was taken as the cut off value for the diluted positive serum sample in comparison to the negative serum control for the determination of final dilution of test serum and the corresponding dilution of the positive sera was found to be 1:800. This dilution was used for all these test serum samples to screen the presence of BTV antibody.

Test proper

Double antibody sandwich ELISA was performed for screening of 220 serum samples collected from cattle of 12 L.B.D. farms of the state according to the method of Western Brink and Kimmam (1987) with slight modification.

1. The anti BTV rabbit immunoglobulin was coated with 50 µl per well (1:400 in coating buffer) on polystyrene microplates (Nunc) and kept at 4°C over night.

2. After adsorption, the plates were emptied and washed three times with PBS tween-20 (pH 7.4).

3. Then the plates were blocked by 3% LAH in PBS tween-20 blocking buffer @ 50 µl quantity in each well and incubated 1 hr at 37°C.

4. Then the plates were washed thrice as per step (2).

5. Then the plates were reacted with 1:3200 dilution of ultra-purified antigen and incubated (50 μl well) for 1 hr at 37°C.

6. Washing was done as per step (2).

7. The test serum samples were added in duplicate wells at a dilution of 1:800 and the plates were incubated at 37°C for 1 hr.

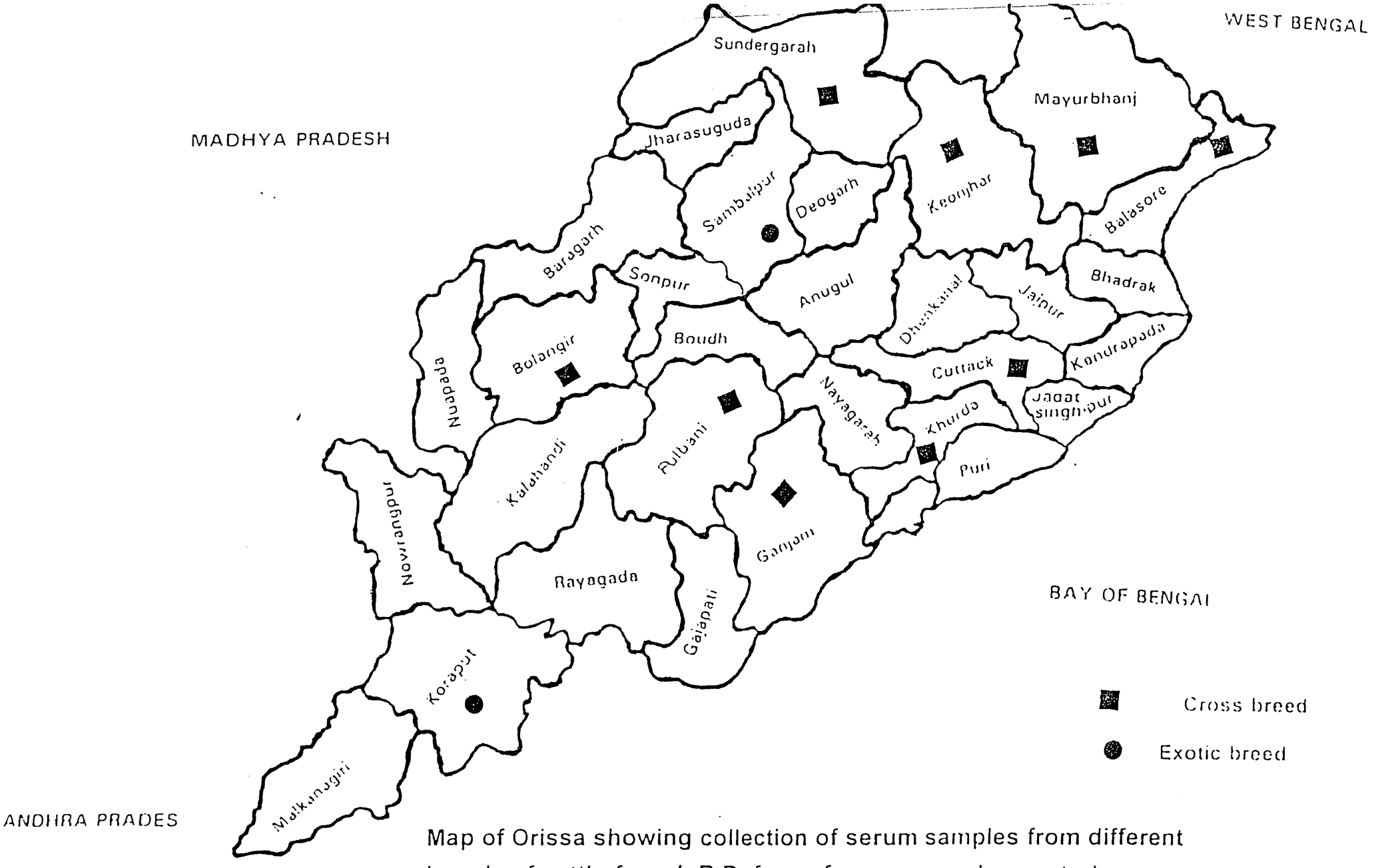
8. Washing was done as per step (2).

9. 50 μl of affinity purified rabbit antibovine conjugate (HRPO labelled Sigma) were added to each well and incubated at 37°C for 1 hour.

10. Following washing thrice with PBS tween-20, 50 μl of ABTS substrate solution was added to each well and incubated for 2 to 5 minutes at 37°C.

11. The reaction was stopped by adding 5% S.D.S. (50 μl/ well) solution.

12. The O.D. was recorded spectrophotometrically at 410 nm in Multiskan ELISA reader (Flow laboratory, USA).



Map of Orissa showing collection of serum samples from different breeds of cattle from L.B.D. farms for seroprevalence study.

CHAPTER-IV

RESULTS

RESULTS

Propagation of BTV in vero cell line and study of CPE

Vero cells were grown into confluent monolayer having a cell concentration of 2 x 10^5 cells/ml with GMEM containing 10% calf serum. The confluently grown monolayer cells were infected with 10^{-2} dilution of reference BTV serotype-10 and observed for the development of cytopathic effect. Following 24 hrs of incubation very few cells were found to be rounded but after 48 hrs of infection, large no. of cells showed rounding, the cells became granular (Fig. 1 and 2). At 72 hrs. post infection, extensive degenerative changes could be found which consisted of large number of enlarged, rounded cells, (Fig. 3) with aggregation of cells and detachment from the glass surface. Complete degeneration of cells and more extensive CPE involving the entire monolayer could be found even after 96 hrs of post infection (Fig 4 & 5). The harvested virus was subjected to 2 cycles of freezing and thawing and passaged 4 times in vero cell line. In each passage, similar cytopathic effects were noticed.

Vero cells grown in coverslip preparation in lighton's tubes were similarly infected with 10^{-2} dilution of stock virus and examined for the microscopic changes by staining with haematoxylin and eosin (H&E) stain at various intervals of infection. After 48 hrs. of infection, majority of cells showed cytoplasmic vacuolation. acidophillic intra cytoplasmic inclusion bodies. swelling and aggregation of rounded cells (Fig. 6 and 7) while control cells remained healthy and did not show any changes.

Titration of the virus

Vero cells grown in confluent monolayers were used in 96 well flat bottomed tissue culture plates were used for titration of the 5th passaged BTV by Reed and Muench formula. Six set of wells, each infected with varying dilution of stock virus ranging from 10^{-1} to 10^{-6} were observed for development of CPE. By 72 hours when CPE became more apparent, the $TCID_{50}$ was determined and found to be $10^{5.2}$/.1 ml.

Physico-chemical properties of virus

Thermostability

The 5th passaged BTV having $TCID_{50}$ of $10^{5.2}$ was subjected to temperature treatment at 45°C,50°C and 56°C for 30 minutes in a water bath and transferred to an ice bath. Such treated inoculum was subjected to titration for the determination of $TCID_{50}$ in cells grown in micro titre plate. Results of $TCID_{50}$ revealed that viruses heat treated at 45°C, had a $TCID_{50}$ of 10^{3}/.1 ml. Which showed a decrease in log 2 $TCID_{50}$. Viruses treated at 50°C had a $TCID_{50}$ less than 1 while viruses treated at 56°C for 30 minutes were completely inactivated as no CPE could be found.

Cationic stabilization

Stock virus inoculum of BTV treated with 1M magnesium chloride and treated at 50°C and 56°C for 30 minutes were subjected to titration which revealed $TCID_{50}$ of $10^{5.2}$/.1 ml in both the treated samples. The result thus confirmed that BTV is stabilized by IM magnesium chloride.

Stability to pH

pH stability showed that stock virus inoculum treated with citrate phosphate buffer when titrated in vero cell found to be labile to the acidic pH as the virus was sufficiently inactivated and did not show any cytopathic effect. Similarly the virus was found to be stable at pH 8.3.

Chloroform sensitivity

Stock BTV cell culture supernatant treated with 10% v/v analytical grade chloroform was also subjected to titration in vero cell and $TCID_{50}$ was found to be $10^{5.2}$/.1 ml which suggests that BTV is resistant to the lipid solvent like chloroform.

Effect of formalin

Stock BT virus being treated with 0.3% formalin when titrated in vero cell, no viral yield was detected because of absence of CPE. This proves that formalin completely inactivated the virus.

Nucleic acid determination

Confluently grown vero cell monolayer was treated with 50 μg of IUDR in maintenance media (M.M) for 1 hr, while control received normal M.M. and incubated for 1 hr. The stock virus at varying dilution (10^{-1} to 10^{-6}) was inoculated into both IUDR treated tubes and control tubes. Titration of both IUDR treated and control tubes revealed a $TCID_{50}$ of $10^{5.2}$/.1 ml respectively. The result clearly indicated that the viral nucleic acid was RNA as it was not inhibited by IUDR.

Growth curve

Vero cells grown in 32 tubes were simultaneously infected with log 10^2 dilution of 5th passage BTV and one set each having two infected tubes were removed at every 8 hrs interval till 96 hrs post infection to study the growth pattern of BTV in vero cell line by titration. The result of the titration in 96 well flat bottomed microtitre plate indicated that at 8hrs post infection (P.I.) there was less than one log titre which gradually increased up to $TCID_{50}$ $10^{3.4}$/.1 ml at 32 hrs post infection and reached the highest peak at 48 hrs post infection with $TCID_{50}$ $10^{5.2}$/.1 ml. There after a gradual fall in the virus titre was noticed and in 96 hrs $TCID_{50}$ was found to be $10^{1.2}$/0.1 ml (Fig. 8). The reading was taken when 75 to 100% of the infected cells showed extensive CPE. Maximum extra cellular virus could be noticed with high multiplicity of infection of (75-100% destruction of monolayers) between 72-96 hrs P.I. whereas a low multiplicity of infection was observed P.I. at 72 hrs with 25 to 100% cell destructions. The growth curve showed that virus yield increases rapidly during first 24hrs post infection approaching the titre of initial inoculum. Although at this stage visible CPE could be found in focal areas.

Serological incidence of BTV

In order to study the sero-prevalence of BTV among cattle of Orissa, a total of 220 serum samples were collected from 12 organised dairy farms of the state. These samples were randomly collected from different age groups, breeds and sexes of cattle which were apparently healthy. The serum samples were screened for the detection of antibodies to BTV through application of polyclonal based sandwich ELISA.

Following the standardization of sandwich ELISA, a cut off value was determined by comparing the mean O.D. value of both positive (1.156) and negative serum (0.076) and it was considered as standard deviation of both positive and negative serum. Any O.D. value exceeding 1.156 was judged to be positive for the test sera. For testing serum samples a particular dilution of serum e.g: 1:800 was selected which showed the minimum O.D. above the cut off value.

Results of sandwich ELISA for defection of BTV antibodies in 220 serum samples of cattle has been depicted in the Table-2.

TABLE 2: SEROLOGICAL INCIDENCE OF BTV IN CATTLE OF ORISSA

Sl. No	Name of the L.B.D. farm	No. of sera tested		No. found positive	% age of positive reactors
		C.B.	Exotic		
1	Khapuria	26	-	17	65.38
2	O.U.A.T.	18	-	11	61.11
3	Remuna	21	-	16	76.19
4	Kathapal	8	-	3	37.50
5	Charbatia	12	-	5	41.66
6	Keonjhar	10	-	7	70.00
7	Chiplima (Sambalpur)	-	41	23	56.09
8	Sundargarah	11	-	6	54.54
9	Bhanja nagar	17	-	11	64.70
10	Bolangir	9	-	5	55.55
11	Phulbani	11	-	7	63.63
12	Sunabeda (Koraput)	-	36	21	58.33
	Total	143	77	132	60.00

Of the 220 serum samples screened for sandwich ELISA for the detection of BTV antibody, 132(60%) revealed presence of BTV antibody (Fig. 9). The percentage of sero-positive reactors ranged from 37% to 76% in various L.B.D farms. The highest percentage of incidence of BTV e.g. was 76% detected in Remuna L.B.D. farm (Balasore) and the lowest was Kathapal L.B.D. farm (Mayurbhanj) where the incidence was found to be 50%. It evident from the above results that BTV is prevalent in almost all government farms of the state which needs careful attention for control of such problems.

Breedwise distribution of BTV antibodies in cattle by sandwich ELISA

The breed wise distribution of BTV antibodies in cattle defected through sandwich ELISA has been presented in the Table 3.

TABLE 3: BREEDWISE DISTRIBUTION OF BLUETONGUE ANTIBODIES IN CATTLE OF ORISSA BY SANDWICH ELISA

Sl.No.	Breed	No. of sera tested	No.of sera positive by sandwich ELISA	% of sera positive by sandwich ELISA
1	Cross breed	143	88	61.53
2	Exotic breed	77	44	57.14
	Total	220	132	60.00%

Perusal of the data shown in the table 3 indicated that out of 143 crossbreed animals samples screened, 88 were positive (61.53%) while of the 77 exotic animals sera screened, 44 (57.14%) were found positive for BTV antibody (Fig. 10). Cross bred animal thus revealed a marginal increase of 4.39% incidence rate of BTV compared to exotic animals.

SEXWISE DISTRIBUTION OF B.T.V. ANTIBODIES BY SANDWICH ELISA IN CATTLE

The sexwise distribution of BTV antibodies in cattle detected through sandwich ELISA has been presented in Table 4.

TABLE 4: SEXWISE DISTRIBUTION OF Bluetongue VIRUS ANTIBODIES BY SANDWICH ELISA

Sl.No.	Sex	No. of sera tested	No. of sera positive by sandwich ELISA	% age of sera positive by sandwich ELISA
1	Male	33	17	51.51
2	Female	187	115	61.49
	Total	220	132	60.00

It is revealed from the above table that sexwise, of the 33 male animals screened serologically, 17(51.51%) were found positive where as 115 female animals out of 187(61.49%) revealed presence of BTV antibody (Fig. 11). The result also revealed incidence of BTV in female animal found to be 9.98% more than male animals.

AGEWISE DISTRIBUTION OF BTV ANTIBODIES BY SANDWICH ELISA IN CATTLE

Age wise incidence of BTV antibodies in cattle detected through sandwich ELISA has been presented on the table 5.

TABLE 5: AGEWISE DISTRIBUTION OF BTV ANTIBODIES BY SANDWICH ELISA IN CATTLE

Sl.No.	Age	No. of sera tested	No. of sera positive by sandwich ELISA	% age of sera positive by sandwich ELISA
1	Below 9 months	54	21	38.88
2	Above 9 months	166	111	66.86
	Total	220	132	60.00%

Analysis of age wise incidence of BTV revealed that of the 54 serum samples obtained from calves (below 9 months), 21(38.88%) were found positive where as of the 166 serum samples from adult animals (above 9 months), 111(66.86%) were found to be positive for BTV antibodies (Fig. 12). These results also indicate that a significant increase of BTV sero-incidence rate 27.99% with adult animal when compared to young animal.

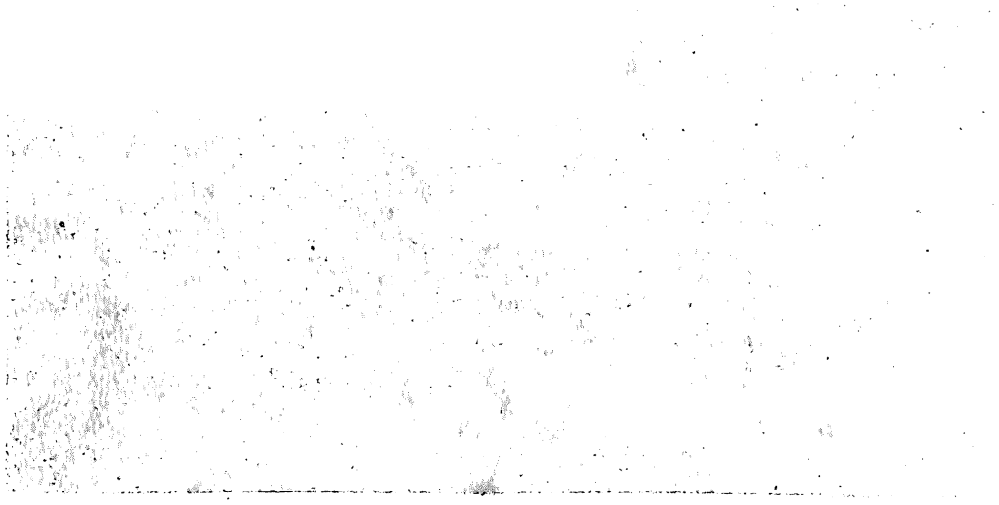


Fig. 1

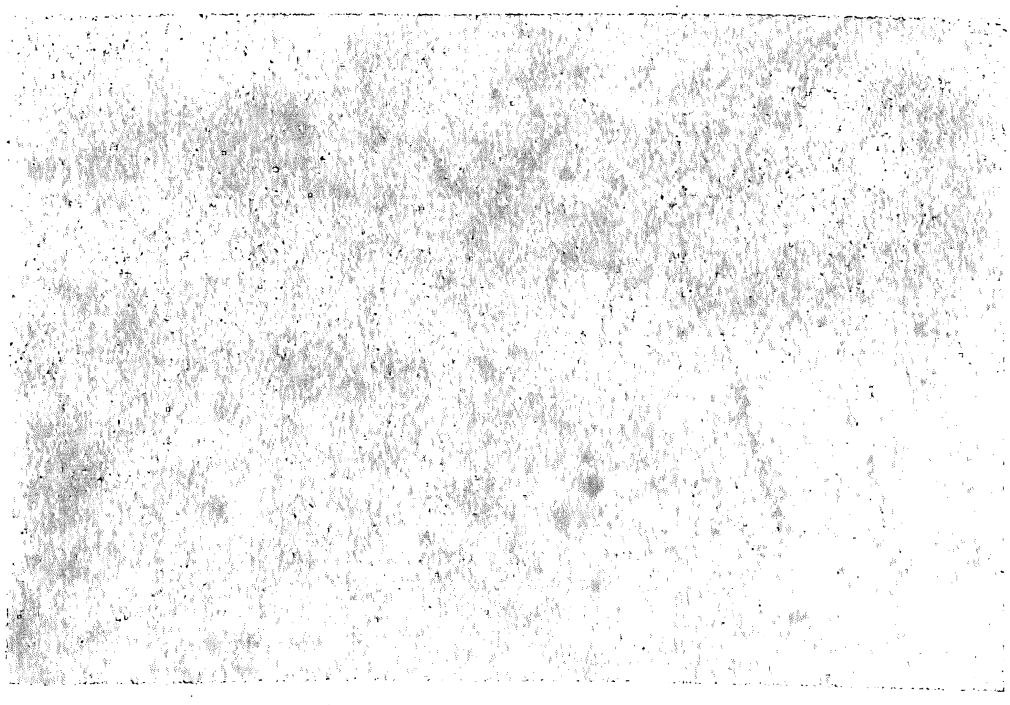


Fig. 2

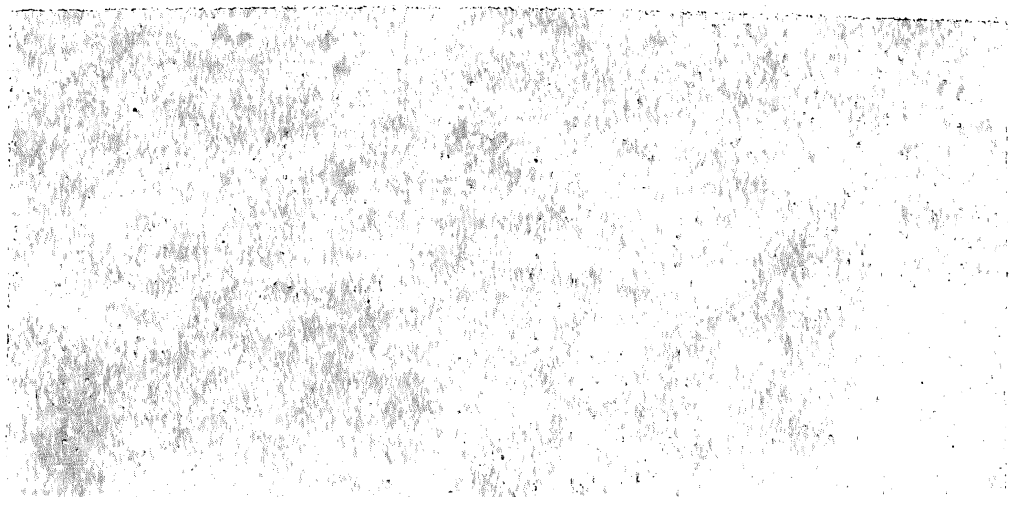


Fig. 3

Fig. 1. Healthy vero cell (x 10) (unstained).

Fig. 2. Vero cell infected with BTV-10 at 5th passage level showing CPE at 48 hrs post infection (unstained).

Fig. 3. Vero cell infected with BTV-10 at 5th passage level showing CPE 72 hrs post-infection (unstained).

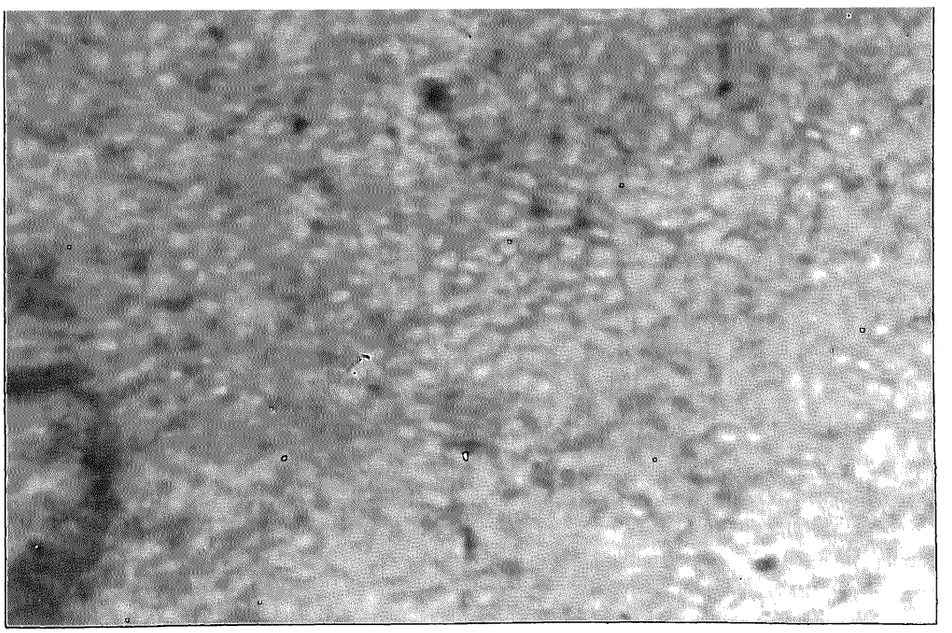


Fig. 1

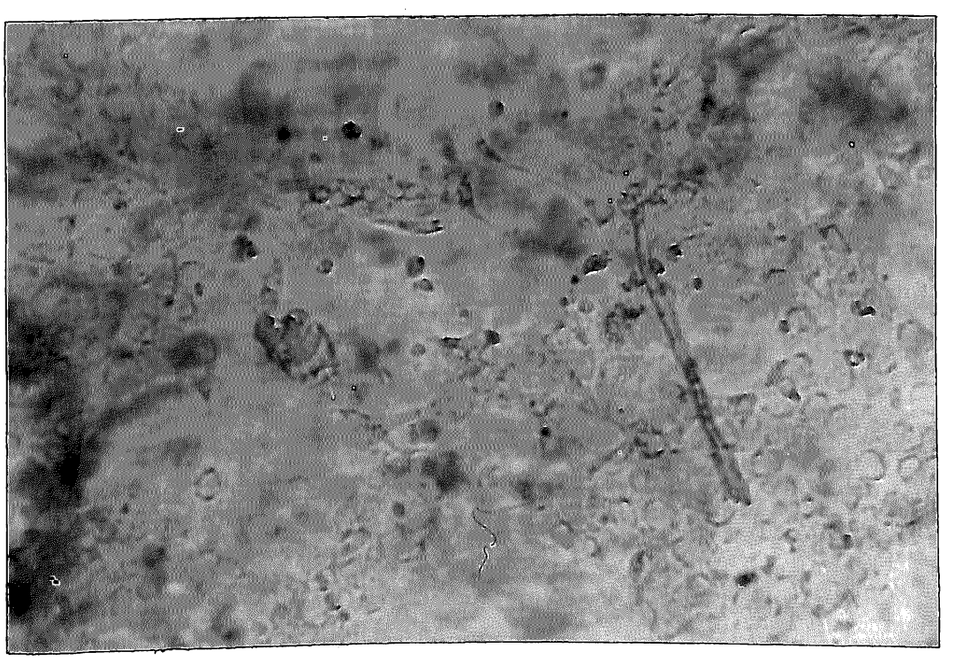


Fig. 2

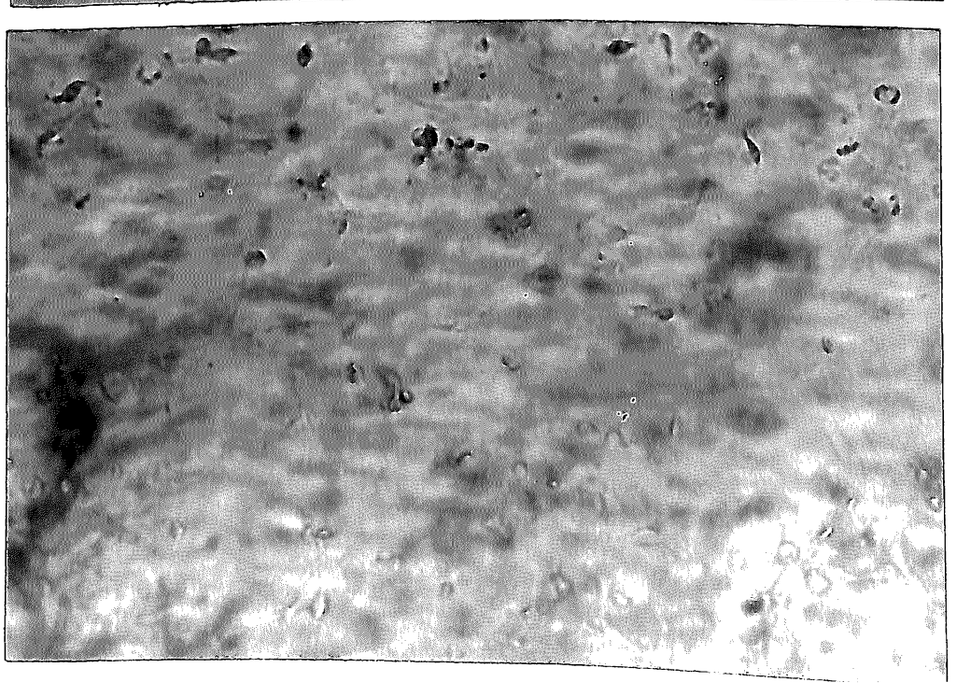


Fig. 3

Fig. 4. Control vero cell stained with H.E. stain at 72 hrs (45x15).

Fig. 5. Vero cell infected with BTV 10 at 5th passage level and stained with H.E. showing characteristic CPE at 48 hrs post infection. (45x15).

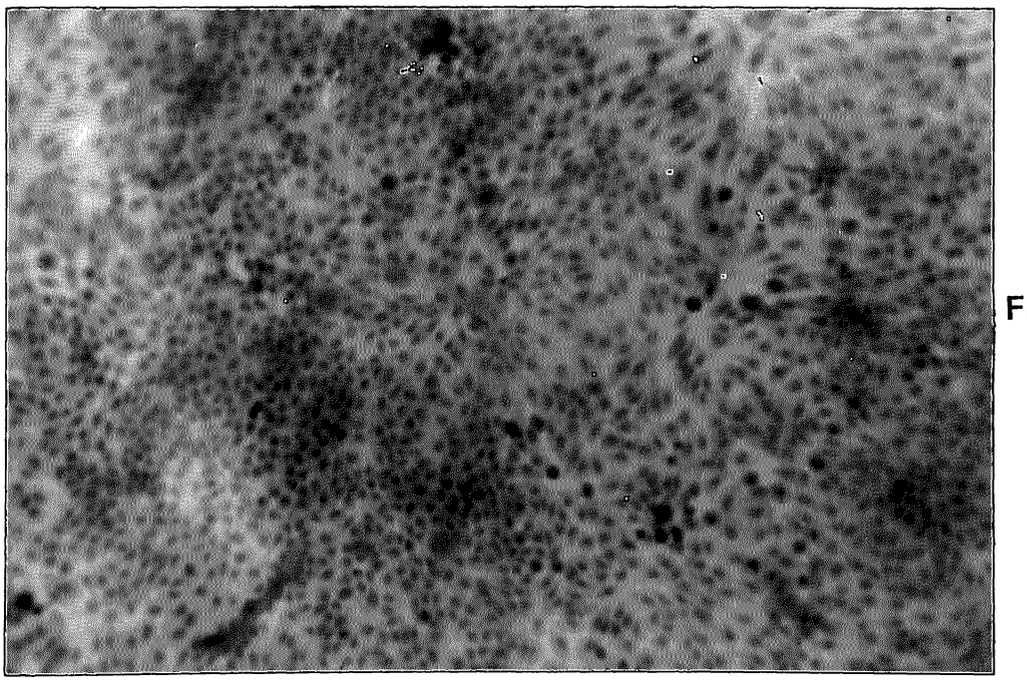


Fig. 4

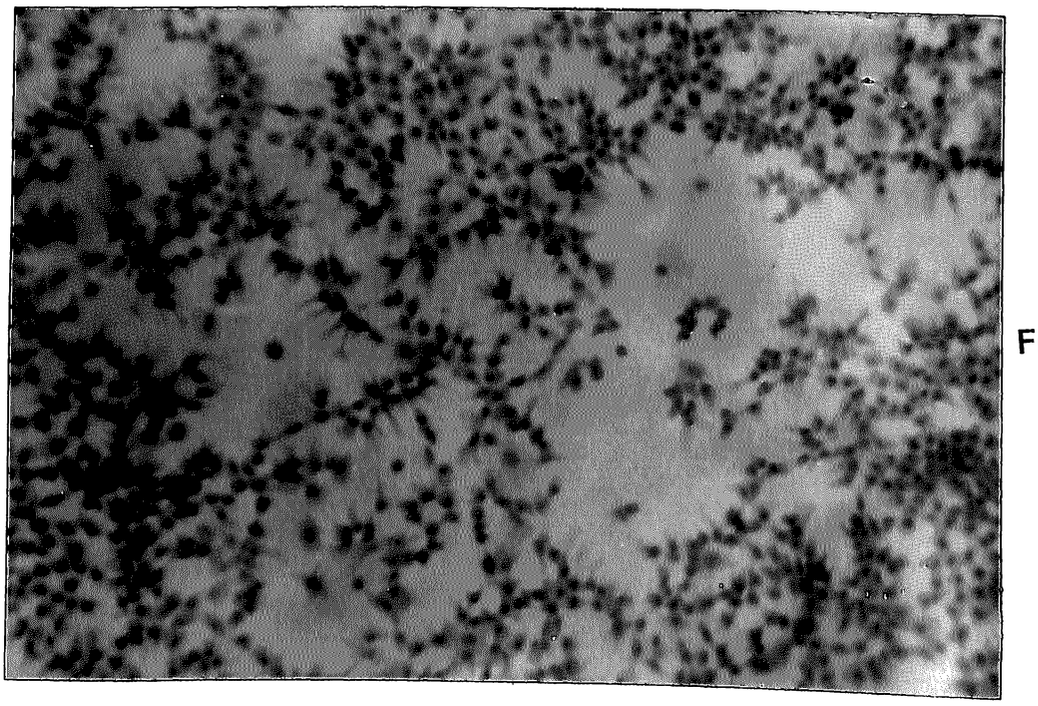


Fig. 5

Fig. 6. Healthy vero cell stained with H.E. (x 100).

Fig. 7. Vero cell infected with BTV-10 at 48 hours post infection showing characteristic intracytoplasmic acidophillic inclusion bodies (x 100).

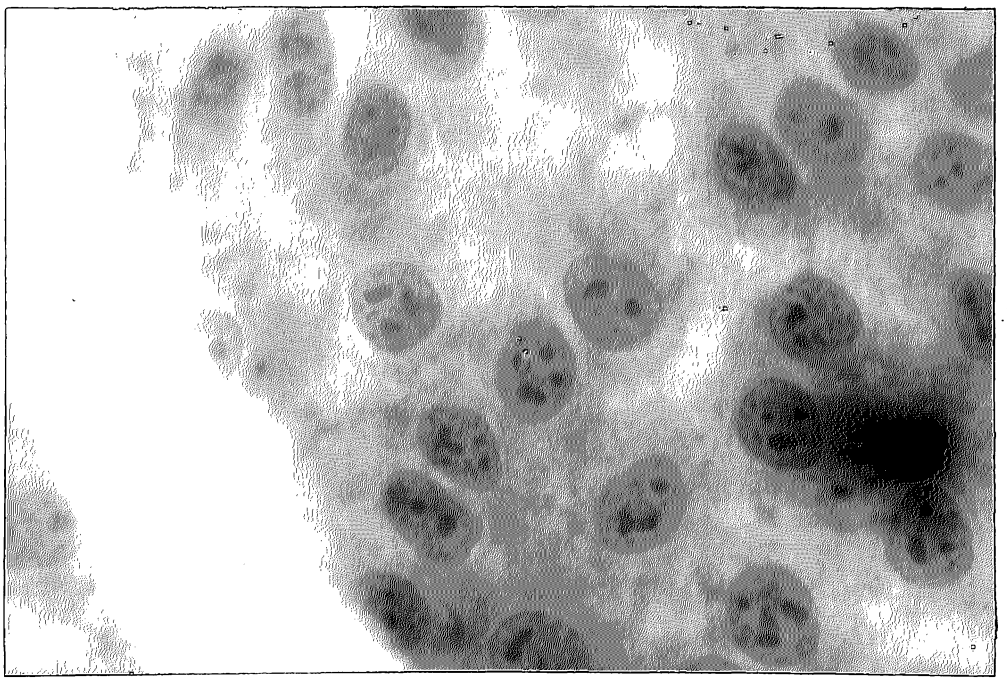


Fig. 6

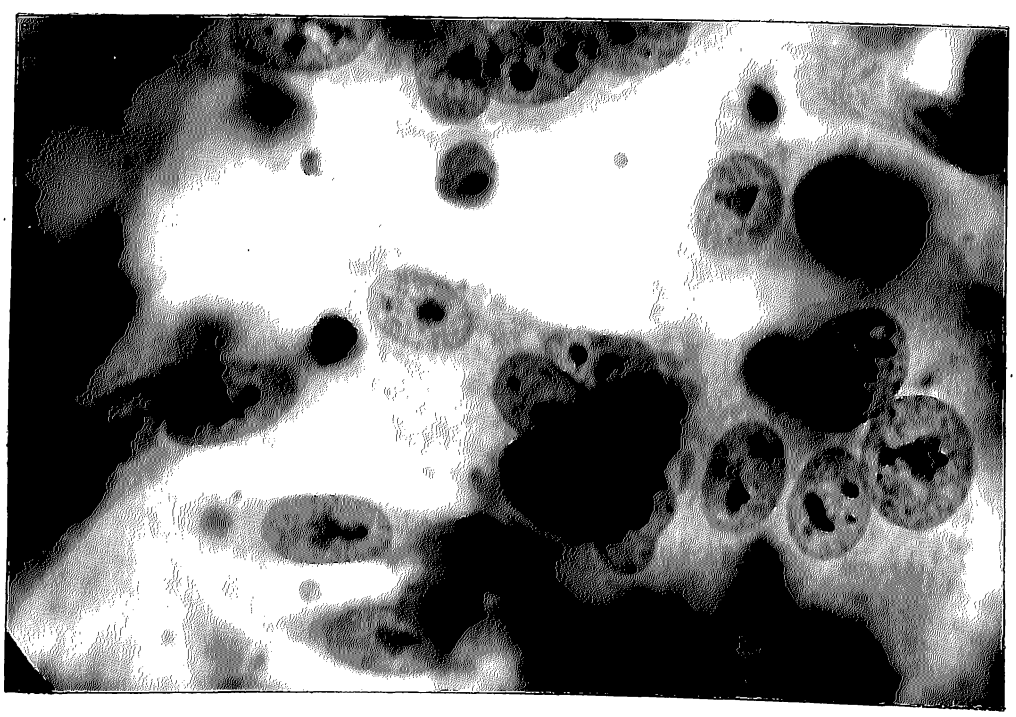


Fig. 7

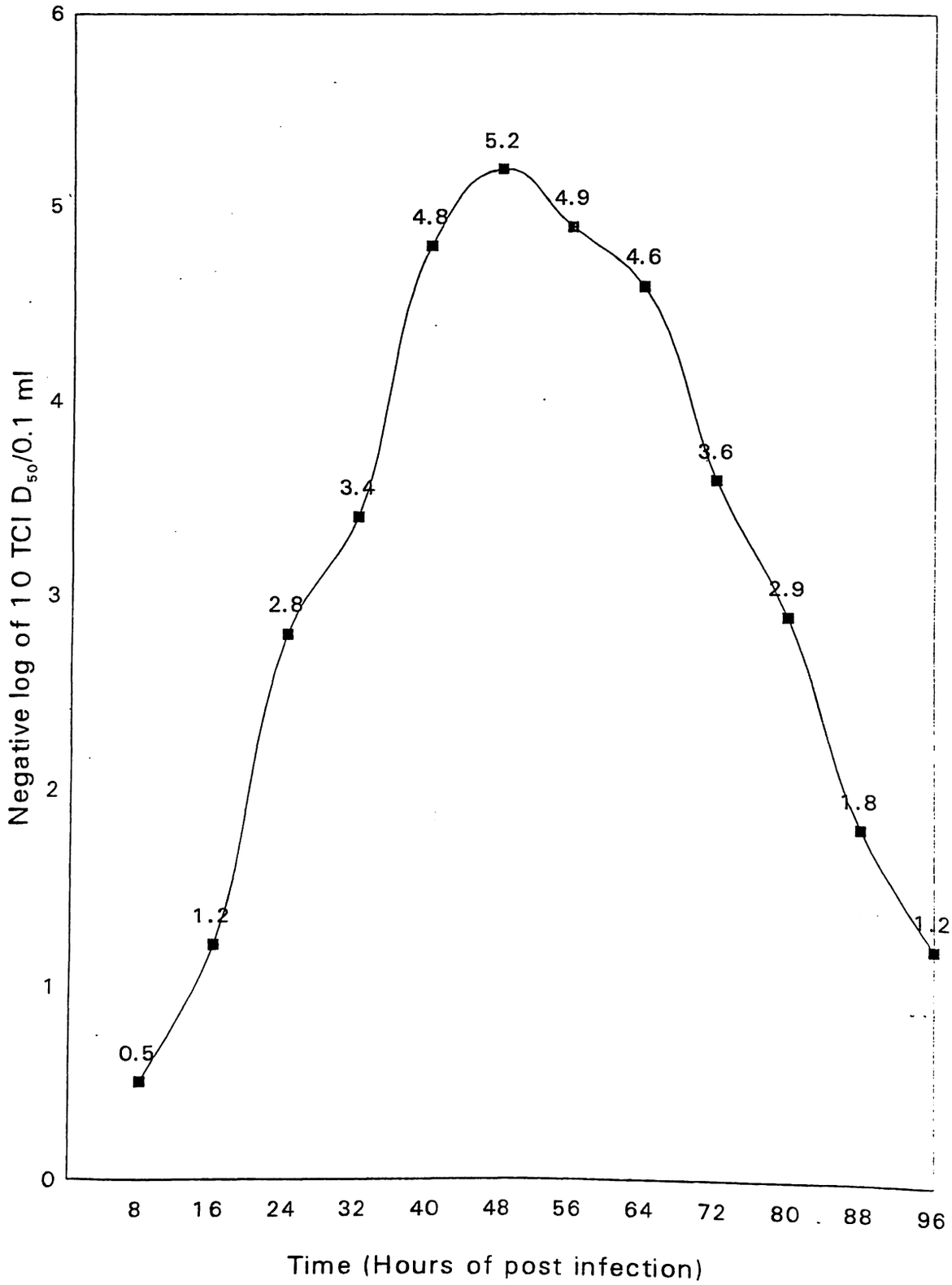


Fig. 8: One step growth curve of BTV-10 in vero cell line

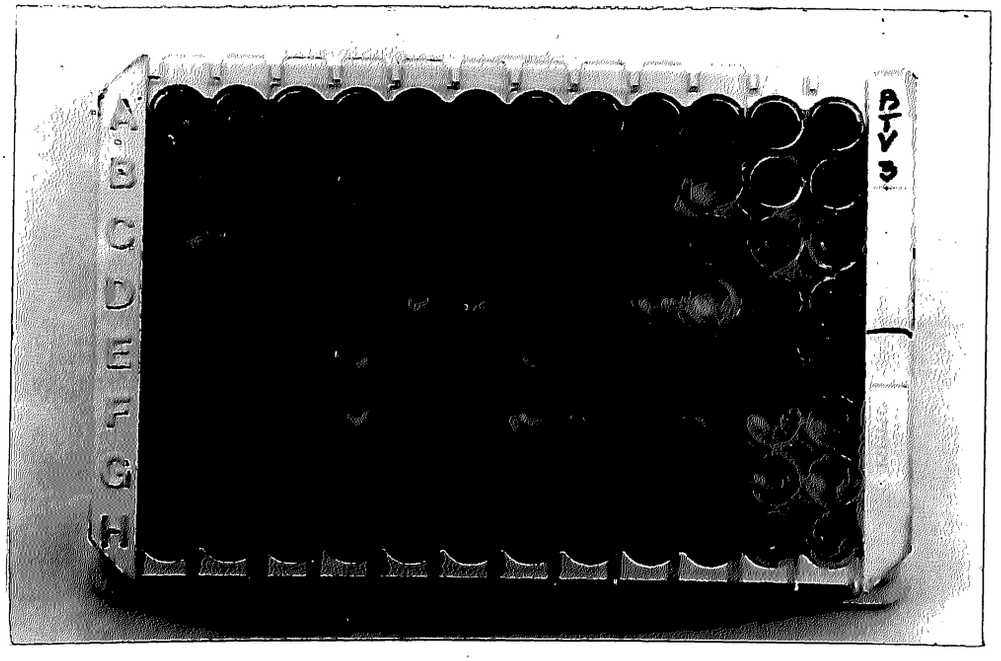


Fig. 9. **Some of the wells in duplicate showing positive chromogenic reaction indicating presence of BTV antibodies in samples of cattle sera. The wells showing less background indicates negative serum samples.**

	Serum samples (in duplicate)										← Control→	
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E											+Ag	+Ab ←
F											+Ag	–Ab
G											-Ag	+Ab
H											-Ag	–Ab

↑

SANDWICH ELISA PLATE LAYOUT

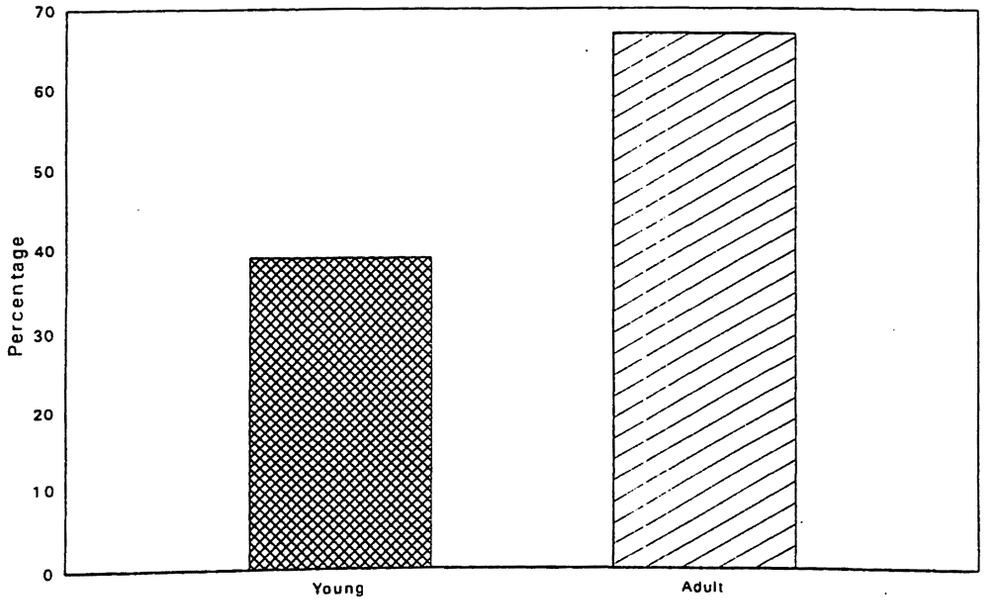


Fig. 12: Figure showing %age of positive reactors in young (below 9 months) and adult (above 9 months) animals by sandwich ELISA for BTV antibody (Table-5)

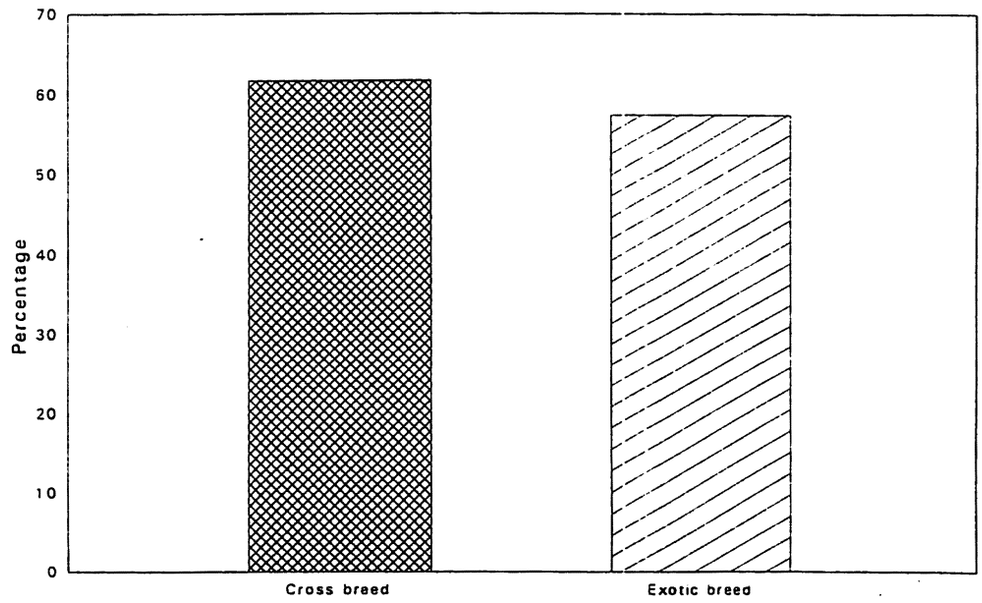


Fig. 10: Figure showing %age of positive reactors in crossbreed & exotic breed cattle by sandwich ELISA for BTV antibody (Table-3)

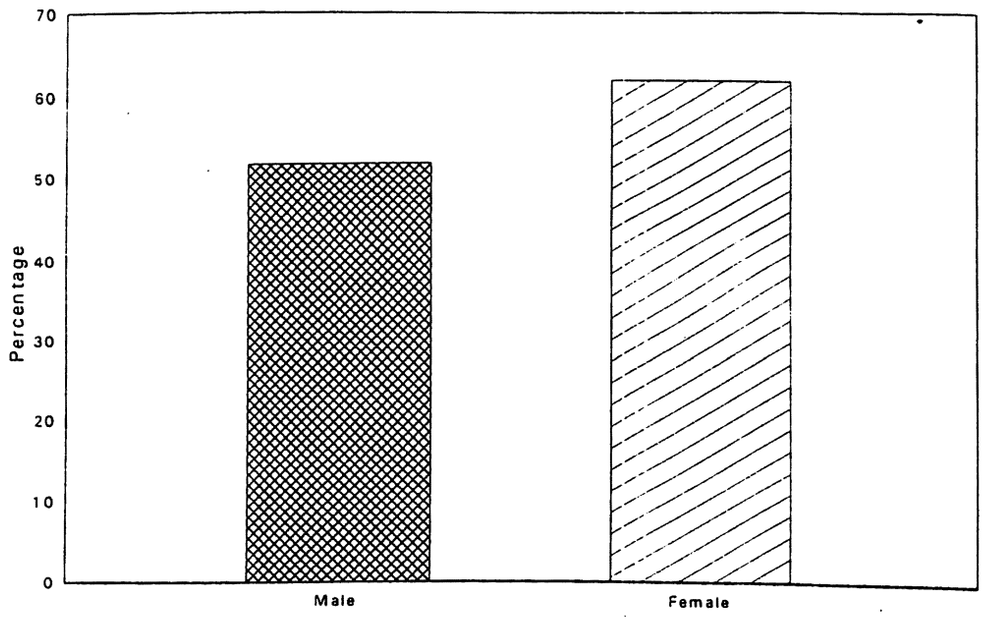


Fig. 11: Figure showing %age of positive reactors in male & female animals by sandwich ELISA for BTV antibody (Table-4)

CHAPTER-V

DISCUSSION

DISCUSSION

Bluetongue being primarily a disease of sheep, has also been reported to cause asymptomatic infection leading to reproductive disorders like abortion, hydroencephalopathy and mummified foetus in cattle. Furthermore, viraemia is prolonged in BTV infected cattle and viraemia continues despite the simultaneous presence of virus specific neutralizing antibodies in sera. Spread of BTV in cattle is mediated by insect vectors like Culicoides species (Luedke *et al.*, 1967) and semen. There are also evidence that BTV overwinter in infected cattle leading to persistance or a sub-clinical type of infection in absence of vector. With the cattle being attributed as reservoir host of BTV, the greatest economic loss in form of reproductive disorder is not only mediated by vector transmission but also by shedding of virus through semen. Such transmission from cattle to cattle also posses a serious threat of spread of infection to other ruminants. Bluetongue virus comprising 24 distinct serotypes with segmented genom also further complicates the diagnostic procedure because of high rate of genetic reassortment leading to large number of variants (Roy, 1996). Such variants have put a hindrance for the development of suitable vaccine for the control of BTV infection in both small and large ruminants. Although serological methods for the detection of group specific antibodies has remained as the major criteria for the diagnosis of BTV, isolation and identification of various serotypes in embryonated eggs and cell culture remains as the ultimate criteria to asses various serotypes involved in a particular outbreak. Isolation of BTV and its identification is mostly based on susceptibility of different cell culture system, physico-chemical

characterization, serotyping by SNT. monoclonal antibody based ELISA and more recently developed serotype specific cDNA probe. It becomes mandatary to study the various physico-chemical properties before proceeding to their identification or to study the various virus-host cell interaction for better understanding of morphogenesis, cyto-chemical changes and the replication cycle all of which may enhance the strategy for a suitable vaccine development.

The present study embodied a detailed physico-chemical and biological characterization of reference BTV type-10 in vero cell line. Propagation of BTV have been earlier attempted by intracerebral inoculation of new born suckling mice (Verwoerd *et al.*, 1979) intravenous, intradermal inoculation of sheep and through intravenous inoculation in embryonated chicken eggs (Alexander, 1947). Propagation of virus in Primary cell cultures like lamb kidney was attempted by Livingstone and Moore 1962, Bando, (1976), through BHK_{21} by St George *et al.* (1978) and by Mc Phee *et al.* (1982) in vero cell. In the present study BTV serotype-10 as a reference strain already adopted in vero was further passaged 5 times in vero cells to study the cytopathic effect. At 24 hrs. post infections, very few cells were found to be rounded where as after 48hrs. of infection, large number of cells showed rounding, granulation, etc. But at 72 hrs post infection, extensive degenerative changes characterised by rounding of more number of cells, granulation, ballooning, aggregation and detachment of cells from glass surface were seen. Following 96 hrs. of infection, a more intense degenerative changes could be found and the cell sheet was almost peeled up. In contrast, Ohder *et al.* (1970) detected CPE after 40 to 72 hrs

of infection. Infected coverslip monolayer stained with haematoxylin and eosin revealed intracytoplasmic inclusion and cytoplasmic vacuolation with empty spaces caused by degenerated cells detachment. Such cytopathic effect in BHK_{21} cells has already been reported by st. George *et al.* (1978, 1980) Walton (1980) Mc Phee *et al.* (1982) in both BHK_{21} and vero cell, Jain *et al.* (1986), Mehrotra *et al.* (1989), Mishra *et al.* (1992) and Xiaocheng *et al.*,(1995)in C_6/36, BHK21 and verocells. However, Deshmukh and Gujar (1999) observed comparatively a late CPE of BTV (96-120 hours) in BHK_{21} cell line. Although various cell culture systems like primary cell cultures and continuous cell lines have been used for various BTV serotypes, calf pulmonary artery endothelial (CPAE) cell line has been found to be most sensitive for BTV propagation (Afshar, 1984).

In the present study, vero cell propagated reference BTV serotype-10 at 5th passage having $TCID_{50}10^{5.2}$/0.1 ml was used for various physico-chemical characterization. In corporation of IUDR into medium for the determination of nucleic acid type indicated the viral nucleic acid to be RNA in nature as it failed to inhibit the replication of the virus. Similar observation was also reported by Verwoerd (1969) who demonstrated that genome of BTV consisted of 10 segments of double stranded RNA like other Reo viruses. Thermostability study by subjecting the reference virus at various temperatures like 45,50 and 56°C for 30 minutes and their titration in vero cell revealed a $TCID_{50}$ of 10^3/0.1 ml at 45° and less than 1 at 50°C whereas virus held at 56°C for 30 minutes found to be completely inactivated. The present finding is in agreement with Wallis *et al.* (1962) and Svehag *et al.* (1963) who observed thermal inactivation of virus at 56°C for

30 minutes was due to protein denaturation and inactivation of RNA respectively. Besides this, the stock virus having a $TCID_{50}$ of $10^{5.2}$/0.1 ml when treated with IM magnesium chloride and held at 50° and 56°C for 30 minutes, did not reveal any fall in titre, thus indicating BTV to be stabilized by IM magnesium chloride. Similar results were obtained by VenDer Walt (1980) who used 2 milli molar tris citrate buffer at (pH 9) Yama Guchi *et al.* (1984) and Inaba *et al.* (1970) who used IM magnesium chloride in their studies. Studies on the sensitivity of BTV to various pH indicated the virus to be labile at acidic pH (pH 3) while it remained stable at pH 7.4. This finding coincides with the observations of Owen (1964), Svehag *et al.* (1966) and Inaba *et al.* (1970). Effect of lipid solvents like chloroform on BTV showed no fall in titre indicating the virus to be resistant to chloroform as has already been reported by Bowne (1971) and Svehag *et al.* (Loc. cit). Virucidal property of certain chemicals like formalin in the given study at a 0.3% concentration was studied for BTV which is in par with the report of Mc Crory *et al.* (1959). Such virucidal agent is thought to act on the nucleic acid by deaminating the nucleic acids and thereby bringing a structural change in the duplex DNA and RNA of viruses rendering the DNA polymerase defunctioned for chain elongation.

Propagation of several BTV serotypes and related orbi virus in embryonated chicken egg and primary cell cultures have been earlier reported by several workers. However, initial isolation of BTV from clinical samples in BHK 21 cells, have been reported by St. George *et al.* (1978 and 1980). In the present study vero cell adopted BTV serotype-10 was further passaged 5 times in vero cell and was used to study the growth

kinetics of the virus. A log 10^{-2} dilution of the virus was used for initial infection and samples collected at each 8 hrs. interval were titrated. The $TCID_{50}$ of the virus was found to be <1 log at 8 hrs P.I. and gradually increased to reach a titre of $10^{-3.5}$/0.1 ml at 38 hrs P.I. and the peak titre of $TCID_{50}$ $10^{-5.2/0.1\ ml}$ at 48 hrs post infection. Thereafter a gradual fall in titre was observed which reached a $TCID_{50}$ of 10^{2}/0.1 ml at 96 hrs post infection. A similar peak titre of log $10^{3.2}$ P.F.U/ml was observed for BTV-20, BTV-1 and BTV strain C.S.I.R.O.-154 by Mc Phee *et al.* (1982) BHK_{21}, VERO, $LLC\text{-}MK_2$ L 29, SVP and Aedes species, Albo pictus cell line. However, the author observed the virus replication was less efficient at higher virus inoculum than at low virus inoculum at 48 hrs post infection which might be due to production of defective interfering (D.I) particle or production of interferon. Highest yield of BTV isolates in BHK_{21}, VERO and L-29 cell lines at 48 hrs P.I. has also been reported by Fernandes, (1959), Bando, (1976), Thomas *et al.* (1976) and also by Mishra (1992), which agrees to the findings of the present study.

Diagnostic procedures for bluetongue involves isolation, identification of virus, detection of virus specific antigens and detection of virus specific antibodies in the serum of convalescent animals. Both a combination of virological and serological procedure is usually employed to detect infectivity in animals. Since, isolation and identification of the causative agent involves extensive use of cell culture system which is neither economic nor time worthy, serological methods score advantage over the former in terms of simplicity and being more economic to perform. Bluetongue virus capsid consists of 2 concentric layers where the outer

layer has 2 major polypeptides VP_2 and VP_5 representing the serotype specific antigen while the inner core has VP_3 and VP_7 represents the group specific antigen. Antigen or antibody to group specific antigen is usually detected by AGPT, CIE, IPT and ELISA. (Met Calm, 1968, Blue *et al.*, 1974, Hafez and Ozawa, 1973, Guindo 1975. Peason 1979, Della-porta *et al.*, 1981, Bandyo Padhyay *et al.*, 1983) while detection of serotype specific antigen or antibody is more relied on the use of SNT and monoclonal antibody based sandwich or competitive ELISA (Barzilai., 1982, Ward *et al.*, 1995, Lage *et al.*, 1996).

However, with the introduction of ELISA, diagnosis of BTV infection in both small ruminants and large ruminants has gained momentum because of its high sensitivity and specificity over the contemporary test. Various modification of the ELISA procedures like direct ELISA, indirect ELISA, IGM captured ELISA, blocking ELISA, polyclonal based sandwich ELISA, monoclonal based sand which ELISA and monoclonal antibody based competitive ELISA have been used by several workers for the sero-diagnosis of BTV. In the present study, a polyclonal based sandwich ELISA has been used to screen 220 serum samples collected randomly from different breeds. sex and age groups of cattle from 12 organised farms of the state. Since sandwich ELISA has already been proved to be more sensitive than indirect ELISA, it was employed in the given study to detect sero-incidence rate of BTV in cattle of Orissa. The results of the test revealed 60% animals (132/220) to be positive for BTV. The breed wise analysis of incidence of indicated that 88 of the 143 crossbred animals sera (61.53%) were positive to BTV while 44 of 77 exotic

animals were found positive (57.14%) for BTV antibody. Hubschle *et al.* (1981) using indirect ELISA could detect BTV antibody as early as 14 days which reached a peak at 42 days post infection. Poli *et al.* (1982) by using indirect ELISA could detect 23% serum sample to be positive which were negative in AGPT. While conducting serological survey of BTV in cattle Odiawal *et al.* (1985) recorded an incidence rate of 32% through ELISA and Afshar *et al.* (1987) used both indirect and competitive ELISA for detection of antibodies to BTV-10 and observed the competitive ELISA to be more superior to detect early antibody response. Drolet *et al.* (1988) in a comparative analysis found that 14% cattle sera negative though AGPT, were found positive by indirect ELISA. Afshar *et al.* (1989) observed the diagnostic specificity of competitive ELISA to be 99.92%, while that of indirect ELISA 99.85% and in AGPT it was 99%. In a large scale seroprevalence study Hugh *et al.* (1989), detected 39% seroincidence rate of BTV in cattle by using indirect ELISA in U.S.A. Singer *et al.* (1997) reported an incidence rate of 89% in cattle using Mab, in comp. ELISA. So there is variation in the serological incidence of BTV, which depends on various factors such as prevalence of the disease, susceptibility of animals environmental factors, roll of vectors and seasonality. Application of blocking ELISA and competitive ELISA has also been used extensively in countries like Canada., U.S.A. U.K. and Australia which was further analysed by Afshar *et al.*, 1981 who proposed that either of the two test systems can be successfully used for seroprevalence study of BTV. Zhow and Chan (1996) used a monoclonal antibody directed against VP_7 antibody, a poly clonal bovine BTV antibody and purified VP_7 antigen for comparing the efficacy of competitive ELISA with that of western blot analysis and observed that 22

serum samples from bovines which were found positive for competitive ELISA, were also positive by western blot.

However, there are scanty reports on the application of indirect, blocking and competitive ELISA for the detection of antibody in cattle in India and there is no report on the application of sandwich ELISA for sero-incidence study of BTV in cattle. Naresh and Prasad, (1995) used a commercial competitive ELISA kit to detect BTV antibody where they could record an incidence rate of BTV to be 60.5% for cattle 78.7% for buffaloes.These observations are in agreement with the present findings. Similar observations were also made by Behera (1997) who recorded 56.072% incidence of BTV in sheep and 34.982% in goats by employing indirect ELISA. Khan (1998) used indirect ELISA for studying the sero-incidence rate of BTV in bovines and found an incidence rate of 68.33% for crossbred animal and 65.55% for exotic animal where as in the present study by using sandwich ELISA, it was found to be 61.53% in cross breed animals and 57.14% in case of exotic animals. This shows that the percentage of positive reactors are comparatively less than the findings of Khan (1998) which may be attributed to the fact that nonspecific binding of bovine antibody to a polyvinyl ELISA plate could have shown an increase of false positive reaction in indirect ELISA Which has been reduced in the present test.

Breedwise, sexwise and agewise analysis of sero-incidence of BTV revealed no appreciable difference which was also reported by Khan (1998).

Perusal from the literature for serological survey of BTV in cattle out lines that indirect ELISA, IGM captured ELISA, blocking ELISA, monoclonal and poly clonal based competitive ELISA score significant results over conventional tests like AGPT, CFT, CIE and SNT. Despite this, the relative superiority of sandwich ELISA and competitive ELISA in terms of sensitivity and specificity for detection of antibodies against many viruses, has already been reported. Due to lack of literature on application of polyclonal based sandwich ELISA from abroad and India, it was felt necessary to undertake studies on the seroprevalence of BTV in cattle through sandwich ELISA which was found more superior to indirect ELISA for detection of antibodies in serum sample. Reports are forthcoming on the application of monoclonal antibody directed against group specific VP_7 bovine antibody through competitive ELISA. Further, diagnosis of BTV infection in small ruminants and large ruminants has gained momentum at present with application of molecular techniques like cDNA probe (Mc Coll and Gould, 1991, Pedley *et al.,* 1998 and Wilson, 1990) which has the capacity to discriminate various strains of the same serotype. It is advocated that such relatively superior test procedures should be applied for effective diagnosis from clinical sample and attempts should be made for development of an immunogenic vaccine against B.T. infection.

CHAPTER-VI

SUMMARY

SUMMARY

1. The present study was undertaken to study the physico-chemical and biological properties of BTV including its sero prevalence in cattle of Orissa.

2. The reference BTV serotype-10 was propagated in vero cell line using GMEM and 10% calf serum up to 5th passage level. The $TCID_{50}$ was found to be $10^{5.2}$ / 0.1 ml.

3. Infected BTV vero cell monolayers showed characteristic CPE like rounding, clumping, granulation and detachment of cells from glass surface and cytoplasmic stranding. Infected vero cell coverslip monolayer revealed cytoplasmic vacuolation and acidophilic intracytoplasmic inclusions at 48 hrs. post infection.

4. Vero cell adopted BTV serotype-10 was used for studying one step growth cycle in vero cell. Titration at each 8 hrs interval revealed peak titre of $10^{5.2}$ at 48 hrs post infection and then followed a decline. The virus yield appeared to increase from 16 hrs P.I. and reached the peak at 48 hrs P.I and showed extensive CPE between 72-96 hrs. P.I.

5. Detailed analysis of the various physicochemical test with BTV revealed that IUDR failed to inhibit viral replication thereby, proving the nucleic acid type to be RNA. The virus was found to be resistant to action of chloroform and sensitive to the action of 0.3% formalin

as it was completely inactivated at 18 hrs exposure. The virus was also found to be labile at acidic pH and stable at pH 7 to 8. Thermostability study revealed complete inactivation of BTV at 56°C for 30 minutes. Similarly virus was found to be stable in presence of IM magnesium chloride.

6. Sandwich ELISA was employed in the present study to detect sero incidence rate of BTV from 220 cattle serum samples collected from 12 organised farms of the state.

7. Of the 220 serum samples examined, 132 were found to be positive by sandwich ELISA for BTV antibody (60%).

8. Study on the sero incidence of BTV in different breeds of cattle indicated 61.53% incidence in cross breed and 57.15% in exotic cattle.

9. Agewise analysis of results revealed that adult animals showed an incidence rate of 66.86% while in young animals it was 38.88%.

10. Sero incidence studies in different sexes revealed that female animals had a incidence rate of 61.49% while it was 51.51% in case of male animals.

CHAPTER-VII

CONCLUSION

CONCLUSION

The given study aims at physico-chemical characterization of BTV serotype-10 using vero cell line and a survey on serological incidence of BTV in cattle of Orissa through sandwich ELISA. The physico chemical test full fills all the criteria of RNA virus and the earlier reported properties of BTV serotype. Such analysis is a foremost requirement not only to study the host cell-virus interaction, adaptability and replication strategy but also required for preliminary identification of field isolate from clinical samples. Sand- wich ELISA comparatively more sensitive than indirect ELISA was standardised and used for sero-incidence studies of BTV. In the present study, a total of 220 sera samples were screened by the above test, which reveals the incidence of BTV to be 60% in cattle of Orissa. In view of the large scale incidence of BTV future research should be directed towards sero-epidemiological studies of BTV in all the breeds of domestic ruminants including wild animals and sentinel herd/ flocks should be established in different regions of the state which will be helpful for monitoring the sero-conversion, seasonality of BTV infection and development of a suitable strategy for control of the disease. Attempts should be made for isolation and identification of different serotypes of BTV circulating in the state for developing a suitable immunogenic vaccines against bluetongue infection. It is also important to develop a suitable, simple, cost effective diagnostic kit employing molecular technique for quick diagnostic of the disease in field conditions.

BIBLIOGRAPHY

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Abu Elzein, E.M.E. (1984). Rapid detection of bluetongue virus antigen in the sera and plasma of camels sheep and cattle in the Sudan. using the gel immunodiffusion test. Archives of virology **79**(½): 131-134.

Afshar, A.; Kayvanfar, H., (1974). Occurance of precipitating antibodies to Bluetongue virus in sera of farm animals in Iran. Veterinary Record. **94**(11): 233-235.

Afshar, A.; Thomas, F.C.; Wright, P.F.; Shapiro, J.I.; Shettigara, P.T.; Anderson, J. (1987). Comparison of competitive and indirect enzyme linked immunosorbent assay for detection of bluetongue virus antibodies in serum and whole blood of clinical microbiology. **25**(9): 1705-1710.

Afshar, A.; Trotter, H.C.; Dulac, G.C. ; Reddington, J.J. (1993) Evaluation of a commercial competitive ELISA test kit for the detection of group specific antibodies to bluetongue virus. Journal of Veterinary Diagnostic Investigation, **5**(3):336-340.

Afshar, A.; Moreau, P.; Dulac, G.C. Thomas, F.C. (1991). Gamma irradiation alters bluetongue virus protein antigen. Acta Virologica. **35**(3): 292-294.

Afshar, A.; Anderson, J.; Eaton, B.T. and Gustafson, G.A. (1991). Serological diagnosis of bluetongue by blocking on competitive ELISA by four laboratories. Journal of Vet. diag. Investigation. (Abstr. Vet. Bull. **62**(2): 4080.

Afshar, A.; Thomas, F.C.; Wright, P.F.; Shapiro, J.L.; Anderson. J. (1989). Comparison of competitive ELISA, indirect ELISA and standard AGID test for detecting bluetongue virus antibodies in cattle and sheep. Veterinary Record **124**(6): 136-141.

Al-Busaidy, S.M.; Mellor, P.S. (1991). Epidemiology of bluetongue and related orbiviuses in the sultanate of Oman. Epidemiology and infection **106**(1): 167-178.

Alexander, R.A.; Haig, D.A. and Adelaar, T.F. (1947). The attenuation of BTV by serial passage through fertile eggs. Onderstepoort. J. Vet. Sci. Anim. Ind., **21**: 231-241.

Anderson, G.A.; Stott, J.L.; Gershwin, L.J.; Osburn, B.I. (1987). Identification of bluetongue virus specific Immunoglobulin E in cattle. Journal of General virology. **68**(9): 2509-2514.

Anno, (1988). Annual Report 1986/1987 of Indonesia.

Apiwatnakorn, B; Bura, P.; Pasavorakul, O. (1995). Serological study for bluetongue in Thailand. ACIAR Proceddings No. **66**.

Afshar, A.; Shakarchi, N.H.; Wright, P.F.; Bosse, B. (1992). Simultaneous screening of bovine sera for antibodies to bluetongue and epizootic haemorrhagic disease of deer viruses by enzyme linked immunosorbent assay. Journal of Veterinary Medicine, Series B. **39**(3): 159-164.

Afshar, A.; Wright, P.E.; Taylor. L.A.; Shapiro, J.L.; Duiuc, G.C. (1992) Development and evaluation of an enzyme linked immunosorbent assay for detection of bovine antibodies to epizootic haemorrhagic disease of deer viruses. Canadian Journal of Veterinary Research. **56**(2): 154-160.

Bando, B.M. and Jones, R.H. (1977a). Proc. Int. Symp. Vet. Lab. Diagn. 1: 361-370. (cited by Thomas, E. Walton, Bull. Off. Int. Epiz., 1980, **92** (7-8): 515-523.

Bando, B.M. (1976). Isolation of bluetongue and epizootic hemorrtagic disease viruses in all culture. In: Proc. Am. Assoc. Vet. Lab. Diagnost; 18th, 1975; pp. 163-174.

Bando, B.M. and Jones, R.H. (1977 b). Cell culture and its application. Eds. Action and Lynn, Academic Press, New York, 667-673.

Bandyopadhyay, S.K.; Mallik, B.B. (1983). Serological prevalence of bluetongue antibodies in India. Indian Journal of Animal Sciences **53**(12): 1355-1356 Vet.Res.Inst.Mukteshwar-Kumaon, Uttar Pradesh 263138. India.

Barzilai, E. (1982). Blue tonge antibodies in camels, Sera in Israel. Refuah Veterinarith **39**(3): 90-93.

Behera, S.N. (1997). Study on serological incidence of bluetongue virus in sheep and goat of Orissa. Thesis submitted to O.U.A.T., Bhubaneswar-3.

Bekker, J.G.; Dekock, G.W. and Quinlan, J.B. (1934). The occurrence and identification of bluetongue in cattle the so called pseudo-foot and mouth disease in South Africa. Onderstepoort. J. Vet. Sci. Anim. Ind., **2**: 393-507.

Bekker, G.L. De Kock, G.W. and Quinlan, J.B. (1934). The occurrence and identification of bluetongue in cattle the so called pseudo-foot and mouth disease in South Africa. Onderstepoort J. Vet. Sci. Anim. Indust. **2**: 393-507.

Bhambani, B.D. and Singh, P.P. (1968). Bluetongue disease in India. Indian Vet. J., **45**: 370-371.

Blue, J.L., Daws, D.L. and Gratzek, J.B. (1974). The use of passive haemagglutination for the detection of bluetongue viral antibodies. Am. J. Vet. Res., **35**: 139-142.

Borden, E.C.; Shope, R.E; Murphy, F.A. (1971). Physicochemical and morphological relationships of some arthropod-borne viruses to blue-tongue virus- a new taxonomic group: Physico-chemical and serological studies. J. Gen. Virol., **13**: 261-271.

Bowne, J.G. and Jones, R.H. (1966). Observation on bluetongue virus in the salivary glands of an insect vector. C. Variipennis. Virology. **30**: 127-133.

Bowne, J.G. and Jochim, M.M. (1967). Cytopathologic changes and development of inclusion bodies in cultured cells infected with bluetongue virus. Am. J. Vet. Res., **28**(125): 1091-1105.

Bowne, J.G.; Luedke, A.J.; Jochim, M.M. and Foster. N.M. (1964). Current status of bluetongue in sheep. J. Am. Vet. Med. Assoc., **144** (7): 759-764.

Bowne, J.G. and Ritchie, A.E. (1970). Some morphological features of bluetongue virus. Virology, **40**: 903-911.

Bowne, J.G.; Luedke, A.J.; Foster, N.M. and Jochim, M.M. (1966). Current aspects of bluetongue in cattle. J. Am. Vet. Med. Assoc. **148** (10): 1177-1180.

Bowne, J.G. (1971). Bluetongue disease. Adv. Vet. Sci. Comp. Med., **15**: 1-46.

Brewer, A.W. and Meclachlan, N.J. (1994). The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. Archives of virology. **136** (3-4): 287-298.

Brookes, S.M.; Hyatt, A.D. and Eaton, B.T. (1993). Characterization of virus inclusion bodies in bluetongue virus-infected cells. J. of Gen. Virol. **74** (3): 525-530.

Burke, C.N.; Luginbuhi, R.E. and Williams, L.F. (1968). Avian adeno like viruses: Characterization and comparision of seven isolates. Avian Dis., **12**: 483-505.

Bussard, A. (1959). Description of technique combining simultaneously electrophoresis and immunological precipitation electro synergesis. Biochem. Biophys. Acta. **34**: 258.

Carrier, S.P.; Boulanger, P. (1975). Improvements in the modified direct complement fixation test and its application in the detection of bluetongue antibodies in cattle and sheep sera. Canadian Journal of Comparative medicine, **39**(2): 231-233.

Chang, Gui Zhu LI, Yamin; Shao Cuili, XU Shi Bai; Zheng Tianran (1995). Epidemiological investigation and control of bluetongue disease in Jiangsu Province. China. ACIAR Proceeding No. 66.

Della-Porta, A.J.; McPhee, D.A.; Wark, C.M.; George, S.T. T.D.; Cybinski, H.D. (1981). Serological studies of two additional Australian bluetongue virus sciates CSIRO 164 and CSIRD 166, Veterinary Microbiology: 166-176.

Della-Porta, A.J.; Sellers, R.F.; Herniman, K.A.J.; Littlejohns, I.R., Cybinski, D.H.; St. George, J.D.; Mc Phee, D.A.; Snowdon, W.A.; Campbell, J.; Cargill, C.; Corbould, A.; Chung, Y.S.; Smith, V.W. (1983 b). Serological studies of Australian and papua New Guinean Cattle and Australian sheep for the presence of antibodies against bluetongue group viruses, Vet. Microbiol. **8**: 147-162.

Deshmukh, V.v. and Gujar, M.B. (1999). Isolation and adaptation of bluetongue virus in cell culture system. Indian J. comp. Microbiol. Immunol. Infect. Dis **20** (1): 14-16.

Drolet, B.S.; Mills. K.W., Belden, E.L.; Mecham, J.O. (1988). Development and application of an ELISA for the detection of antibody of BTV. Proceeings of the United States Animal Health Association **92**: 113-122.

ELS, H.J. and Verwoerd, D.W. (1969). Morphology of bluetongue virus. Virology, **38**: 213-219.

Engvall, E.; Pearlmann, P. (1972). Enzyme linked immunosorbent assay ELISA (III). Quantification of specific antibodies by enzyme labelled anti-immunoglobulin in antigen coated tubes. J. Immon **109**: 129-135.

Fenner, Frank. I.; Gibbs, E. Paul; Murphy, Frederick. A.; Rott, Rudolf; Studdert, Michael. J.; White David. O. (1987). Veterinary Virology (2nd edition) 542-543.

Fernandes, M.V. (1959). Isolation and propagation of bluetongue virus in tissue culture. Am. J. Vet. Res., **20**: 398-408.

Fernandes (1959) described that 10 cell lines including Hela HB_2 clone were susceptible to BTV.

Foster, N.M. (1969). Personal communication, U.S. Department of Agriculture, Animal Disease Research Laboratory, Denver, Colorado.

Fulton, R.W.; Burge, I.J.; Cummins, J.M. (1989). Neutralizing antibody responses to bluetongue and epizootic haemorrhagic diseases virus serotypes in beef cattle. American Journal of Veterinary Research **50**(5): 651-654.

Fulton, R.W.; Nich0lson, S.S.; Pearson, N.J.; Potter, MT; Archbald, L.F.; Pearson, J.E.; Jochim, M.M. (1982). Bluetongue infections in louisiana cattle. American Journal-of-Veterinary-Research **43**(5):887-891.

Gambles, R.M. (1949). Bluctongue of sheep in Cyprus. J. Comp. Path. Ther., **59**: 176-190.

Girad, A.; Ruckerbauer, G.M.; Gray, D.P., Bannister, G.L. and Boulanger, P. (1967). Studies on bluetongue iv. studies of 3 strains in primary bovine foetal kidney cell culture. Can. J. Comp. Med. Vet. Sci. **31**: 171-174.

Govt. of Orissa; A.R.D. Deptt. (1995-96) District Profile on Livestock Census 3-4.

Guindo, O (1975). Bluetongue serological survey of domestic and wild ruminants in tropical Africa. (IVRI, Computer Network).

Gupta, Y. (1988). Isolation of serological characterisation of bluetongue virus. M.V.Sc. Thesis submitted to Haryana Agriculture University, Hissar, India, cited by Mahesh *et al.* Bluetongue in Indian perspective.

Gupta, Y.; Chand, P.; Singh, A. and Jain, N.C. (1990). Dot immunobinding assay in comparision with ELISA for detection of BTV antibodies in sheep. Vet. Microbiol., **22**(4): 365-371.

Hafez, S.M. (1978). Serological survey of bluetongue in Iraq. Bulletin de l'office Inter National des Epizooties. **89**(½): 13-22 Vet. Lab. Abu Ghraib, Baghdad, Iraq.

Hafez, S.M.; Ozawa, Y. (1973). Serological survey of bluetongue in Egypt. Bulletin of Epizootic. Diseases of Africa 21 No.3. 297-304 Near East Anim. Hith. Inst. Serum and Vaccine Inst., Abbassia, Cairo.

Haig, D.A.; Mc kercher, D.G. and Alexander, R.A. (1956). The cytopathogenic action of BTV in tissue cultures and its application to the detection of antibodies in the serum of sheep. Onderstepoort. J. Vet. Res., **27**: 171-177.

Hardy, W.T. and Price, D.A. (1952). Sore muzzle of sheep. J. Am. Vet. Med. Assoc., **120**: 23-25.

Haynes, T.B.; Harrold Susan, Schultz, D.R. (1982). A seroepidemiologic survey of antibody to bluetongue virus in Alabama cattle. Cornell vet. **72**: 262-268.

Hefez, S.H.; Taylor, W.P. (1985). Serotypes of B.T. virus present in Saudi Arabia. 531-537. Progress in clinical and Biological Research Vol. **78**.

Homan, E.J.; Ruiz. H.L.D.E.: Donato, A.P.; Taylor, W.P.; Yuill. T.M. (1985). A preliminary survey of the epidemiology of B.T. in Costa Rica and Northern Colombia. Journal of Hygiene **94**(3): 357-363.

Howell, P.G. (1960). A preliminary antigenic classification of strains of bluetongue virus. Onderstepoort. J. Vet. Res., **28**: 357-364.

Howell, P.G. and Verwoerd, D.W. (1971). Bluetongue virus "Virology monographs", **9**: 37-74.

Howell, P.G. (1963). Bluetongue. In "Emerging Diseases of Animals". 61, 111-153. Food and Agriculture organization of the United Nations, Department of Veterinary Services. Onderstepoort, South Africa.

Hubschle, O.J.B.; Lorenz, R.J.; Matheka. H.D. (1981). ELISA for detection of BTV antibodies American Journal of Veterinary Research, **42**(1): 61-65.

Hugh-Jones, M.E.; Taylor, W.P.; Jones, F.; Luther, G.; Miller, J.; Karns, P.; Hoyt, P. (1989). Serological observations on the epidemiology of bluetongue infection in Louisiana cattle. Preventive Veterinary Medicine **7**(1): 11-18

Hutcheon, D. (1981). Fever of epizootic catarrah. Report from the college of veterinary surgery, 1980, p. 12.

Inaba, Y.; Tanaka, Y.; Ishii, S.; Morimoto, T., Sato, K., Omori, T. and Matumoto, M. (1970). Jap. J. Microbiol., 14: 351 (cited by Inaba, Y. Aust. Vet. J. Vol. 51, April, 1975).

Inaba, Y.; Tanaka, Y.; Ishii, S.; Morimoto, T. Sato, K.; Omori, T. and Matumoto. M. (1970). Jap. J. Microbiol., 14: 351 (cited by Inaba, Y. Aust. Vet. J. Vol. **51**, April, 1975).

Jain, N.C; Sharma. R.; Prasad, G. (1986). Isolation of bluetongue virus from sheep in India. Vet. Rec., **199**: 17-18.

Jain, N.C.; Prasad. G.; Gupta, V. and Mahajan, N.K. (1988). Isolation of bluetongue virus from culicoides sp. in India. Rev. Sci. Tech. Off. Int. Epiz., **7**: 375-378.

Jochim, M.M. and Chow, T.L. (1969). Immuno diffusion of bluetongue virus. Am. J. Vet. Res., **30**: 33-41.

Katoch, R.C.; Sambyal, D.S. (1991). A preliminary survey of bluetongue virus precipitating antibodies in livestock of Himachal Pradeh. Indian Vet. Med. J., **15**: 308-310.

Khan, A.A. (1998). Serological incidence of bluetongue virus in cattle of Orissa. Thesis submitted to O.U.A.T.; Bhubaneswar-3.

Komarov, A. and Goldsmit, L. (1951). A BT like disease of cattle and sheep in Israel. Refuah Veterinarith, **8**: 96-100.

Lage, A.P.; Castro, R.s.; Melo, M.I.V.; Aguiar, P.H.P.; Barreto Filho, J.B.; Leite, R.C. (1996). Prevalence of antibodies to bluetongue bovine herpes virus 1 and bovine viral diarrhoea. mucosal disease viruses in water bufaloes in Minas Gerais state, Brazil, Veterinary Bulletin **49**(3): UFMG.

Lecatsas, G. (1968). Electron microscopy study of the formation of bluetongue virus. Onderstepoort. J. Vet. Res., **35**: 139-150.

Liendo, G.; Castro, A.E. (1981). Bluetongue in cattle: Diagnosis and virus isolation. Bovine practitioner No. 87-95.

Livingston, C.W. and Moore, R.W. (1962). Cytochemical changes of bluetongue virus in tissue cultures. Am. J. Vet. Res., **23**(95): 701-710.

Lonkar, P.S.; Uppal, P.K.; Belwal, L.M. and Mathur, P.B. (1983). Bluetongue in sheep in India. Trop. Anim. Hlth. Prod.; **15**: 86.

Luedke, A.J. (1970). Distribution of virus in blood components during viraemia of bluetongue. Proc. Ann. Meet. US. Anim. Hlth. Assoc., 74.

Luedke, A.J. (1969). Bluetongue in sheep: viral assay and Viraemia. Am. J. Vet. Res.; **30**: 499-509.

Luedke, A.J.; D.V.M.; (1967). M.S.; Jochim, M.M., Jones, R.H. (1969). Bluetongue in cattle: Viremia Am. J. Vet. Res. Vol. **30**(4): 511-516.

Lunt, R.A.; White, J.R. and Blacksell, S.D. (1988). Evaluation of a monoclonal antibody blocking ELISA for detection to bluetongue virus in experimental and field sera. Journal of General virology, **56**(11): 2729-2740.

Maggona-Vougiouka, O.; Nomikou. K.; Mastroyianni-Korkolopoulou, M. (1988). Comparative study of the ELISA and the AGID test in the diagnosis of bluetongue.

Manso-Ribeiro, J. Rosa-Azevedo, J.A., Noronha, F.D.; Braco Forte, M.C.Jr.; Grave Pereira, C. and Vasco-Fernandes, M. (1957). Bluetongue in Portugal. Bull. Off. Int. Epiz., **48**: 350-367.

Mc Crory, B.R.; Foster, N.M. and Bay, R.C. (1959). Virucidal effect of some chemical agents on bluetongue virus. Am. J. Vet. Res., **20**: 665-669.

Mc Coll, K.A. and Gould, A.R. (1991). Detection and characterization of bluetongue virus using the polymerase chain reaction. Virus Research. **21**: 19-34.

Mc Phee, D.A.; Parsonson, I.M. and Della-Porta A.J. (1982). Comparative studies on the growth of Australian bluetongue virus serotypes in continuous cell lines and embryonated chicken eggs. Vet. Microbiol., **7**: 401-410.

Mehrotra, M.L.; Shukla, D.C. and Kataria, J.M. (1989). Isolation and identification of viral agents resembling bluetongue virus in an outbreak in sheep. Indian J. Anim. Sci., **59**: 1072-1074.

Mehrotra, M.L. and Shukla, D.C. (1990). Seroprevalence diagnosis and differential diagnosis of bluetongue virus disease in India. Indian J. Virol. **12**: 98-103.

Mehrotra, M.L. and Shukla, D.C. (1984). Sero prevalence of bluetongue in sheep, cattle and buffaloes in India. Indian. J. Anim. Sci., **54**: 718-719.

Met Calf, H.E. (1968). Results of serological survey in wild life in united states (cited Aust. Vet. J. **51**: 205).

Metcalf, E.; MPH; DVM; Pearson, E.; James, MS. DVM; Klingsporn, L.Albert, DVM (1981). Bluetongue in cattle: A serologic survey of slaughter cattle in the United States. Am. J. Vet. Res. Vol. **42** (6): 1057-1981.

Micheael, P.; Ward, Tim, E.; Carpenter, Bennie I. Osburn, (1994). Host factors affecting seroprevalence of bluetongue virus infections of cattle American J.Vet.Res. Vol. **55**(7): 916-920.

Mishra, S. and Mehrotra, M.L. (1996). Physico-chemical and biological characterization of an Indian isolate of bluetongue virus. Indian. J. Comp. Microbiol. Immunol. Infect. Dis. **17**(2): 109-113.

Naresh, A.; Prasad, G. (1995). Relative superiority of C-ELISA for detection of bluetongue virus antibodies. Indian Journal of Experimental Biology. **33**(11):880-882.

Neitz, W.D. (1948). Immunological studies on bluetongue in sheep. Onderstepoort. J. Vet. Sci. Anim. Ind., **23**: 93-136.

Oberoi, M.S.; Gurkipal-Singh and Kwatra, M.S. (1988). Serological evidence of bluetongue virus activity in cattle and buffalo population. Indian J. Virol. **4**: 50-51.

Odiawa, G.; Blue, J.L.; Tyler, D.E.; shotts, E.B. (1985). Bluetongue and epizootic haemorrhagic disese in ruminants in Georgia: American Journal of Veterinary Research **46**(10): 2193-2196.

Odiawa, B.; Blue, J.L.; Tyler, D.E. and Shotts, E.B. (1985). Bluetongue and epizootic haemorrhagic disease in ruminants in Georgia, surveyed by serotest and virologic isolation. Am. J. Vet. Res. **46**(10): 2193-2196. (Abst. Vet. Bull. **56**(2): 853.

Ohder, H. Lund, L.J. and White land, A.P. (1970). Observations on the growth and development of bluetongue, Nairobi sheep disease and Rift valley fever viruses by fluorescent antibody technique and titration in a tissue culture system. Arch. Ges. Virusforsch., **29**: 127-138.

Omori, T. (1961). Fievre, Catarrhaledue mouton (Bluetongue). Bull. Off. Int. Epiz., **50**: 1109-1117.

Owen, N.C. and Munz, E.K. (1966). Observations on a strain of bluetongue virus by electron microscopy. Onderstepoort. J. Vet. Res., **33**(1): 9-14.

Owen, N.C. (1964). Investigation in the pH stability of virus and its survival in mutton and beef. Onderstepoort. J. Vet. Res., **31**: 109-118.

Pearson, J.E.; Carbrey, E.A.; Gustafson, G.A. (1985). Bluetongue and related orbovirus diagnosis in the united states 469-475 [Progress in clinical and biologicl research volume 178).

Pedley, S.; Mohammed, M.E.H. and Mertens, P.P.C. (1988). Analysis of genome segments from six different isolates of bluetongue virus using RNA - RNA hybridisation: a generalised coding assignment for bluetongue viruses. Virus. Res., **10**: 381-390.

Pini, A. (Cackley, W. and Ohder, H. (1966). Arch. Ges. Virusforsch, 18: 89 (cited by Inabe, Y. Aus. Vet. J. Vol. 51, April, 1975).

Poli, G.; Stott,J.L; Liu, Y.S; Manning, J.S. (1982). Bluetongue virus: Comparative evaluation of Enzyme-linked Immunosorbent Assay, Immunodiffusion and serum Neutralization for detectionof viral Antibodies. Am. J. Vet. Res. **42**(1): 159-162.

Polson, A. (1948). Onderstepoort. J. Vet. Res. 23: 137. (cited by Inaba, Y. Aust. Vet. J., Vol. **51**, April, 1975).

Prasad, G.; Jain, N.C.; Mahajan, N.K. and Vasudevan, B. (1987). Prevalence of bluetongue precipitating antibodies in different domestic animals. Ind. J. Anim. Sci., **57**: 552-554.

Rahman, A.H. and Manickam, R. (1997). Toxorhynchites-fluorescent antibody system for the defection of bluetongue virus from culicoides midges. Onderstepoort. J. of veterinary Res. **64**(4): 301-307.

Sapre, S.N. (1964). An outbreak of blue-tongue in goats and sheep in Maharastra state. Ind. Vet. Rev. M & B, **15**: 69-71.

Sarwar, M.M. (1962). A note on bluetongue in sheep in West Pakistan. Pakist. J. Anim. Sci., **1**: 1.

Sendow, I.; Daniels, P. (1992). Application of ELISA to detect bluetongue virus group infection. Seminar held on 27-28 October in Bogon, Indonesia entitled ELISA. Application in Veterinary Science, Penyakit, Hewan. **25**(46A): 68-71.

Shapiro, J.L.; Wiegers, A.; Dulac, G.C.; Bouffard, A.: Afshar, A.; Myess, D.J.; Dubue, C.; Martin, M.W.; Koller, M. (1992). Candian Journal of Vet. Research (1991) **55**(2): 203-204.

Sharma, S.N. (1981). Trop. Anim. HLth. and prod. 13.

Simpson, V.R. (1979). Bluetongue antibody in Botswana's domestic and game animals. Tropical animal health and production **11**(1): 43-49.

Singer, R.S.; Jessup, D.A.; Gardner, T.A.; Boyoe, W.M. (1997). Pathogen exposure patterns among sympatric populations of bighorn sheep, mule deer and cattle,Journal of Wildlife Diseases. **32**(2): 377-382.

Smith, P.C.; Potgieter, L.D.N. New, J.C.; Hall, R.F.; Patton, C.S. (1981). Bluetongue virus infection in Tennessee Cattle. Coll. Vet. Med. Univ. Knoxville, Tennessee.

Sodhi, S.S.; Oberoi, M.S.; Sharma, S.N. and Baxi, K.K. (1981). Prevalence of bluetongue precipitating antibodies in sheep and goats in Punjab, Indian Zbl. Vet. Med., **28**: 421-423.

Song. K.C. (1969). The immune response to and a serological survey for the bluetongue like virus in Korean cattle, Res. Rep. off. Nur. Dev. Suwon, Korean 12, No.5.cited vet. bulletin, **4**: (11).

Spreull, J. (1902). Report from Vety. Surgeon Spreull on the result of his experiments with the malarial catarrhal fever of sheep. Agril. Journal from the cape of Good Hope., **20**: 469-477.

St. George, T.D.; Standfast, H.A.; Cybinski, D.H.; Carley, J.G.; Fillippich, C. and Frazier. C.L. (1978). The isolation of a bluetongue virus from culicoides collected in the Northern Territory of Australia, Aus. Vet. J., **54**: 153-154.

St. George, T.D. Cybinski, D.H.; Della-Porta, A.G.; Mc Phee, D.A.; Work, M.C. and Banbridge, M.H. (1980). The isolation of 2 bluetongue viruses from healthy cattle in Australia. Aust. Vet. J. **56**: 562-563.

Stott. J.L.; Osburn, B.I.; Bushnell, R.; Loomis, E.C.; Squire, K.R.E. (1985). Epizootological study of bluetongue virus infection in California livestock; 571-582 [Progress in clinical and biological research volume 178].

Stott. L.: Jeffrey. Myra. Blanchard-Channell, B.S.; Bennie. I. Osburn, DVM, H.P.: Riemann. DVM. R.C.: obeso. DVM. (1989) serologic and virologic evidence of bluetongue virus infection in cattle and sheep in Mexico. American Journal of Vet. Res. **50**(3), 335-340.

Stott, J.L., Barber. T.L. and Osburn, B.I. (1978). Serotyping bluetongue virus, a comparision of plaque inhibition (disc). and plaque neutralization methods. Proc. 21st Ann. Meet. Am. Assoc. Vet. Lab. Diagn., 29-31 Oct. 399-410.

Studdert, M.J. (1965). Sensitivity of bluetongue virus to ether and sodium deoxycholate. Proc. Exp. Biol. Med. **118**: 1006-1009.

Svehag, S.E. (1963). Thermal inactivation of bluetongue virus. Arch. Ges. Virusforsch, **13**: 499-510.

Svehag, S.E.; Leendertsen. L. and Gorham, J.R. (1966). Sensitivity of bluetongue virus to lipid solvents, trypsin and pH changes and its serological relationship to arboviruses. J. Hyg. (camp). **64**: 339-346.

Tamayo, R.; Schoebitz, R.; Alonso, O.; Wenzel, J. (1985). First report of bluetongue antibody in chile. 555-558 progress in clinical and biological research volume 178.

Teclaw, R.F.; Mc Conell , S.; Wagner, G.G.; Romo, S.; Garcia, Z. (1985). Serologic study of the incidence and prevalence of bluetongue infections in cattle in the mexican states preventive veterinary medicine **3**(5): 437-443.

Theiler, A. (1906). Bluetongue in sheep Ann. Rept. Dir. Agric. Transvaal for 1904-1905 pp. 110-121.

Theiler, A. (1908). The inoculation of sheep against bluetongue and the results in practice. Vet. J., **64**: 600-607.

Thomas, F.C.; Girard, A.; Boulanger, P. and Ruckerbauer, G. (1976). A comparison of some serological tests for bluetongue virus infection. Can. J. Comp. Med., **40**: 291-297.

Tongaoker, S.S.; Ayanger, S.K.; Singh, B.K. and Kant, R. (1983). Sero prevalence of bluetongue virus in Indian buffalo (*Bubalis bubalis*). Vet. Rev.; **112**: 326.

Uhaa, I.J.; Riemann, H.P.; Thurmond, M.C.; Franti, C.E. (1990). A cross sectional study of Bluetongue virus and Mycoplasma bovis infections in dairy cattle. Veterinary Research Communications. **14**(6): 461-470.

Uhaa, I.J.; Riemann, H.P.; Thurmond, M.C.; Franti, C.E. (1990). A seroepidemiological study on bluetongue virus in dairy cattles in the Central Valley of California. Veterinary Research Communication, **14**(2): 99-112.

Uppal, P.K. and Vasudevan, B. (1980). Occurrence of bluetongue in India, Ind. J. Comp. Microbiol. Immunol. Infect. Dis. **1**: 18.

Van Der Walt, N.T. (1980). A haemagglutination and haemagglutination inhibition test for bluetongue virus. Onderstepoort. J. Vet. Res., **47**: 113-117.

Verwoerd, D.W.; Huismans, H. and Erasmus, B.J., (1979). Orbiviruses. In: H. Fraenkel conrat and R.R. Wagner (Editors) comprehensive virology 14: Newly Characterised Vertebrate Viruses. Plenum Press, New York, pp. 285-345.

Verwoerd, D.W. (1969). Purification and characterization of bluetongue virus. Virology, **38**: 203-212.

Verwoerd, D., Louw, H. and Ollermann, R. (1970). Characterization of bluetongue virus nucleic acid. J. Virol. **5**: 1-7.

Wallis, C.; Yang, C.S. and Melnick, J.L. (1962). Effects of cations on thermal inactivation of vaccinia, Herpes Simplex and Adeno viruses. J. Immunology, **89**: 41-46.

Walton, T.E. (1980). The diagnosis and control of bluetongue. Bull. Off. Int. Epizootic., **92**: 515-523.

Ward, M.P.; Flanagan, M.; Baldock, F.C. (1995). Infection of cattle queensland with bluetongue viruses. Australian Veterinary Journal.

Ward, M.P.; Gardner, I.A.; Flanagan. M. (1995). Evaluation of an agargel immunodiffusion test to detect infection of cattle with bluetongue viruses in queensland Australia. Veterinary Microbiology Australia. **45**: 27-34.

Ward, M.P.; Carpenter, T.E. (1995). Infection of cattle in Queensland with bluetongue viruses: Distribution and antibodies Australian veterinary journal **72**(6): 217-220.

Wechsler, S.J.; Mc Holland, L.E. (1988). Susceptibilities of 14 cell lines to bluetongue virus infection. J. Clin. Microbiol., **26** (11): 2324-2327.

White, J.R. (1994). Validation of a quantitative ELISA for comparison of monoclonal antibody affinities for isolates of bluetongue virus. Journal of Immunological methods, **177**(1-2): 79-88.

Wilson, W.C.; Fukosho, A. and Roy, P. (1990). Diagnostic complementary DNA probes for genome segments 2 and 3 of epizootic haemorrhagic disease virus serotype-1. American Journal of Veterinary Research. **516**: 855-860.

Xiaocheng, L.I. Shium, Yanxia, Cheng, Zhongguo, Cheng, Guangxias, Meng, Weihan, Zhou and Genyuansu (1995). A strain of virus isolated from *Culicoides homotomus*. Bluetongue disease in South-east Asia and the pacific. ACIAR Proc. No. 66 Page 90.

Yama guchi, S.; Imada, T.; Kawamura, H. Taniguchi, S.; Saio, H. and Shiwamatsu, K. (1984). Outbreak of egg drop syndrome 1976 in Japan and its etiological agents. Avion Dis., **26**: 626-641.

Yang, C.Y. (1986). A study on P-ELISA in diagnosis of blue-tongue. Animal Husbandry and Veterinary Medicine, **18**(1):2-3.

Yunlong, B.; LI Chundi; LI Shiyin; Qing B.O.; Zhong Nan; Hojinfeng; Yang Ruilan (1995). An epidemiological survey of bluetongue in Yunnan province China. ACIAR proceedings No.**66**.

Zhow Enmin; Chan, M.K.H. (1996). Immunoblot assay as a confirmatory test of competitive ELISA for serodiagnosis of bluetongue. Journal of Veterinary Diagnostic Investigation Canada, **8**(3): 365-367.

APPENDIX

All the solutions were prepared in triple glass distilled water and all the chemicals used were of AR/GR grade.

Cell culture media and solution:

Growth medium (GM):

Glasgow modified essential medium (GMEM)

Ingredients	g/l
NaCl	6.300 g
KCL	0.400 g
$CaCl_2$	0.260 g
$MgSO_4.7H_2O$	0.200 g
$Na_2HPO_4.2H_2O$	0.130 g
D-Glucose	4.500 g
Glutamine	0.300 g
TPB	3.000 g
Phenol red	3 ml
Amino acids	50 ml
Vitamins	4 ml
$NaHCO_3$	2.200 g
Calf serum	100 ml
Distilled water	to make 1000 ml

This was sterilized by filtration through Seitz EKS filter pad and stored at 4°C.

Maintenance media (MM):

Glasgow modified essential medium free of calf serum was used for preparation of virus inoculum and for maintenance of cell monolayer after infection.

Trypsin-Versene solution:

Ingredients	g/l
NaCl	8.000 g
KCl	0.400 g
KH_2PO_4	0.200 g
Na_2HPO_4	1.900 g
$NaHCO_3$	1.000 g
D-Glucose	0.500 g
Trypsin (1:250)	1.700 g
EDTA	1.400 g
Phenol red	1 ml
Distilled water	to make 1000 ml

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

Reagents for ELISA:

Coating buffer (Carbonate-Bicarbonate buffer pH 9.6):

Sodium carbonate	0.318 g
Sodium bicarbonate	0.586 g
Sodium azide	0.040 g

The volume was made upto 200 ml with distilled water.

Washing buffer (PBS-Tween 20, pH 7.4):

Sodium chloride	8.0 g
Potassium chloride	0.200 g
Disodium hydrogen orthophosphate	1.445 g
Potassium dihydrogen phosphate	0.200 g
Sodium azide	0.200 g
Tween-20	0.500 ml
Distilled water	1000 ml

Phosphate citrate buffer (pH 5.0):

A.	Citric acid	1.921 g
	Distilled water	100 ml
B.	Disodium hydrogen phosphate	2.840 g
	Distilled water	100 ml

Mix 24.3 ml of A and 25.7 ml of B and volume made upto 100 ml with distilled water.

Blocking buffer:

PBS pH 7.2	100 ml
LAH	3 gm
Tween-20	0.05 ml

Buffers:

Phosphate buffer saline (PBS, pH 7.2):

Sodium chloride	8.000 g
Potassium chloride	0.200 g
Disodium hydrogen phosphate	2.312 g
Potassium dihydrogen phosphate	0.200 g
Distilled water	1000 ml

PBS (pH 7.4) was prepared as per the above mentioned ingredients.

Hank's balanced salt solution (HBSS, pH 7.4):

Hank's solution I:

Sodium chloride	80 gm
Potassium chloride	4 gm
Magnesium sulphate	2 gm
Calcium chloride	1.4 gm
Distilled water	1000 ml

Sterilized at 15 lb pressure for 15 min in an autoclave.

Hank's solution II:

Disodium hydrogen phosphate	1.2 gm
Potassium dihydrogen phosphate	0.6 gm
Dextrose	20 gm
Phenol red solution (0.04%)	50 ml
Distilled water	950 ml

Sterilized at 15 lb pressure for 15 min in an autoclave.

Hank's solution III:

Sodium bicarbonate	7.0 gm
Distilled water	500 ml

Sterilized through seitz filtration.