ISOLATION AND MOLECULAR CHARACTERIZATION OF CHICKEN INFECTIOUS ANEMIA VIRUS

Dissertation

Submitted to Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
VETERINARY MICROBIOLOGY
(Minor Subject: Animal Biotechnology)

By

Sreekala S. Mohandas
(L-2014-V-11-D)

Department of Veterinary Microbiology
College of Veterinary Science

Ludhiana – 141 004

2019

CERTIFICATE – I

This is to certify that the dissertation entitled, **"Isolation and molecular characterization of chicken infectious anemia virus"** submitted for the degree of **Ph.D.** in the subject of **Veterinary Microbiology** (Minor Subject: **Animal Biotechnology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Sreekala S. Mohandas (L-2014-V-11-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

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

_____

(Dr. P. N. Dwivedi)
Major Advisor
Professor cum Head
Deptt. of Dairy Microbiology
College of Dairy Science and Technology
Guru Angad Dev Veterinary and Animal Sciences University,
Ludhiana-141004 (Punjab)

CERTIFICATE – II

This is to certify that the dissertation entitled, **"Isolation and molecular characterization of chicken infectious anemia virus"**submitted by **Sreekala S. Mohandas (L-2014-V-11-D)**, to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfilment of the requirements for the degree of **Ph.D.** in the subject of **Veterinary Microbiology** (Minor Subject: **Animal Biotechnology**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an external examiner.

(Dr. P.N. Dwivedi)
Major Advisor

(Dr. D. Muthuchelvan)
External Examiner
Principal Scientist,
Division of Virology, I.V.R.I.
Mukteswar Campus 263 138,
Nainital (Distt.), Uttarakhand

(Dr. T.S. Rai)
Head of the Department

(Dr. Sanjeev Kumar Uppal)
Dean, Postgraduate Studies
Guru Angad Dev Veterinary and
Animal Sciences University,
Ludhiana, Punjab

ACKNOWLEDGEMENT

The blossoming of my life and fruit reaped in this harvest season are only possible with those in my life who persevered and toiled their compassion, love, selfless dedication for a man's growth and maturation. ***"Gratitude"*** *is the best attitude and there is no more pleasing exercise of the mind than gratitude. I consider it as a proud privilege and feel immense pleasure to acknowledge all those who helped me during the pursuit of study. Through this piece of acknowledgement, I would like to extend my heartfelt regards and gratitude towards all those who always stand by me through the ups and downs of this itinerary and whose help is indispensable for the completion of this manuscript. First and foremost, I would like to thank my greatest teacher of all: Supreme Power of Universe. I know that I am here and that I am able to write all of this for a reason. I will do my best in never forgetting what a great fortune I have had in just being here, and that it comes with a lesson and a responsibility. I hope I am doing the work you have planned me to do.*

I take this opportunity to express my esteemed and profound sense of gratitude and regard to my reverent guide, ***Dr. P. N. Dwivedi****, for his affability, worthy guidance, valuable suggestions, unreserved help, constant encouragement, gentle and caring attitude throughout the period of my research work. His able guidance, ingenious advice, constructive counsel, inspiration, patient help made real contribution for the consummation of this task. I will always remember his positive attitude, always looking ahead tendency, that he gave during this study had brought the best out of me. I successfully overcame many difficulties and learned many technical and practical skills that I will take with me throughout the rest of my academic and professional journey. Thank you, sir.*

I am highly thankful to co-advisor ***Dr. Gurpreet Kaur,*** *Assistant Professor, Department of Veterinary Microbiology, for her understanding, encouragement supportive attitude and personal attention which have provided good and smooth basis for my Ph.D. I am also grateful to her for providing necessary infrastructure and resources to accomplish my research work. Thank you, Madam. I gratefully acknowledge* ***Dr. Chanchal Singh,*** *Assistant Professor, Department of Veterinary Physiology and Biochemistry, for giving guidance, support and valuable suggestion during my work.* ***Dr. Ramneek,*** *Director, School of Animal Biotechnology, for his constructive suggestions, benevolent criticism, and all the coordination work when I needed it most.* ***Dr. A.K Arora,*** *Professor (Dean PGS Nominee), Department of Veterinary Microbiology, for his scholastic guidance, constant inspirations, diligent help and all sorts of co-operation during my research work.*

I express my deep sense of gratitude to ***Dr T. S. Rai****, Professor cum-Head, Department of Veterinary Microbiology for his valuable suggestions, constant inspiration and providing me necessary facilities to carry out this work in the department. Words are compendious to express my sincere thanks to the faculty members of my department* ***Dr. N. S. Sharma, Dr. H. M. Saxena, Dr. Deepti Narang, Dr. Mudit Chandra, Dr. Paviter Kaur*** *and* ***Dr. GursimranFilia*** *for their sincere help, moral support, constant encouragement and constructive suggestions*

My profound thanks are due to ***Dr. Neeraj Kumar Singh*** *and* ***Dr Dipak Deka****, Assistant scientist from School of Animal Biotechnology for letting me encroach upon their time and experiences freely in the form of technical help, answering my queries during the most critical phases.*

Most of the results described in this thesis would not have been obtained without the help of few laboratories. I owe a great deal of appreciation and gratitude to professor cum Head, Department of Pathology, Department of Veterinary Physiology and Biochemistry, Department of

Livestock Production Management, Clinical Diagnostic Laboratory (TVCC), Department of Animal Breeding and Genetics

I warmly remember ***Venkateshwara Hatcheries*** *Pvt. Ltd. in Pune (India) forever willing help rendered by them during my research work.*

It has been a great privilege for me to acknowledge to the ***Director Research,*** *of GADVASU,for providing necessary facilities which buttressed me to perform my research work comfortably. I am thankful to* ***Dean PGS and Dean COVS****, GADVASU, for providing necessary facilities and resource required for this work.*

I am thankful to ***Department of Science and Technology (DST),*** *Govt. of India for granting the project and financial support under* ***Women Scientist Scheme (WOS-A)*** *to carry out this work. I would also like to take this opportunity to thank DST for financial assistance in the form of WOS-A fellowship, which was imperative for completion of my Degree.*

Special thanks to my friends, juniors and seniors Dr(s). ***Rakhi ma'am, Anju, Monika, Ajay, Shalini, Lavina, Mamta, Najeeb, Rabyia, Neha, Kirandeep, Karanvir, Satpal, Gurleen, Amandeep, Derhasar, Prabhakar, sugandha, Jagruti, Shaswati, Himashri, Pallvi, Jasleen, Ravneet, Sankalp, Losa Rose, Asiya, Sai Lakshmikanth, Tirathraj*** *and* ***Sahil Sharma,*** *for their constant support, encouragement and good times spent together.*

I acknowledge the valuable support from the office and laboratory staff ***(Ms. Ravneet, Ms. Raman, Sh.Uday, Sh. Amit, Sh. Ram Dev Yadav, Sh. Lal Babu, Sh. Ranjit, Sh. Prem, Sh. Harjeet, Sh. Sunil and Ms. Satnam,****), Department of Veterinary Microbiology, is gratefully acknowledged.*

I would like to thank everybody who was important to the successful realization of this thesis, as well as expressing my apology that I could not mention personally one by one.

Words sound hollow and phrases lose their meaning when heart is full of sentiments and my vocabulary utterly fails in expressing my accolade to my revered parents ***P.K, Vijayakumari, S.N. Mohandas,*** *the motivation and constant encouragement given by them has made many difficult tasks easier for me and supported my mind. I am thankful to god for blessing me with sisters* ***Sreeja*** *Chechi and* ***Sreevidhya****, Brother-in-Law* ***Prasanth*** *Chettan who have been very helpful and kind enough to listen to my concerns during the course of study. Mere words can't explain my feelings to father in-law* ***Sohanlal Kaswan****, mother in-law* ***Guddi Devi,*** *brother in-laws* ***Vineeth Kaswan*** *and* ***Dinesh Kaswan*** *for their moral support and, encouragement. Special mention to my cute little angels* ***Achu*** *and* ***Unni****, I am very proud of you and wish you only the best in life and throughout your future endeavors.*

I wish, If I could find words which can express my feelings of indebtedness, respect, love and regards for my dearest son ***Shaurya*** *and husband* ***Sandeep Kaswan****, your encouragement when the times got rough are much appreciated and duly noted. It was a great comfort and relief that you tried to provide all the necessary things timely to complete my work. Thank you so much.*

Date:

Place: *Sreekala S Mohandas*

Title of Dissertation	:	Isolation and molecular characterization of chicken infectious anemia virus
Name of the Student	:	Sreekala S. Mohandas
Admission No.	:	L-2014-V-11-D
Major Subject	:	Veterinary Microbiology
Minor Subject	:	Animal Biotechnology
Name and Designation of the Major Advisor	:	Dr. P. N. Dwivedi Professor
Degree to be Awarded	:	Ph.D.
Year of award of Degree	:	2019
Total pages in Dissertation	:	99 + VITA
Name of University	:	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141 004, Punjab, India

ABSTRACT

Chicken infectious anemia (CIA) is an emerging immunosuppressive viral disease of chicken. The present study was planned to know the serological evidence, detection of chicken Infectious Anemia virus (CIAV) circulating among the flocks and genetic characterization of isolated CIAV from various regions of poultry dense Punjab state. Serological study was conducted by indirect ELISA and presence of virus in seropositive birds was detected by Polymerase chain reaction (PCR) and was genetically characterized. The presence of CIAV was also confirmed in tissues samples *viz.* Thymus, Liver, Bone marrow, Spleen, Bursa of dead birds which showed characteristic signs and symptoms of CIA. Further, isolation of the CIAV from field tissue samples was done in Marek's disease virus transformed lymphoblastoid cell line. The isolated virus was molecularly characterized and phylogenetic relationship was analyzed by various bioinformatic tools. Hematological parameters were estimated from collected blood samples. In the sero-molecular study, chickens of various age group (n=120) from different flocks (n=27) and different geographical areas (n=12) were tested for evidence of seroconversion to CIAV. Majority of the samples were from apparently healthy birds (both layer and broiler) but few clinically ill birds also included in the present study. Sixty-six (55%) birds were found positive for anti-CIAV antibody. Fifty-one (56.04%) of the apparently healthy birds tested were also found positive for CIAV antibody. There was significant decrease in packed cell volume, haemoglobin and erythrocyte count among seropositive birds than seronegative ones. Majority of the seropositive birds (74.24%) were found positive for presence of CIAV genome. Sequencing and bioinformatic analysis of the most conserved gene (VP3) showed 100% homology with various other reported Indian and world isolates of CIAV. Tissue samples of dead birds (n=65) from various regions (n=10) of Punjab state had presence of virus in almost all the lymphoid organs with highest quantum in thymus tissues. Thirty-nine (60%) birds were found positive for genome of CIAV by PCR method. All the three important viral genes (VP1, VP2 and VP3) screened for confirmation of the amplified genome as of CIAV showed presence of virus in the dead birds. Birds found positive for CIAV genome by PCR were further used for isolation of CIAV in cell line (MDCC MSB1). Ten isolates of CIAV were obtained from positive field samples. Confirmation of the cell culture isolate was done by immunoassays to demonstrate viral antigens and PCR for demonstration of CIAV genome. The whole genome of the all cell culture isolates was amplified by PCR having approximately 2.3Kb amplicon after resolving in electrophoresis. Sequence obtained show high similarity with various Indian and global isolates reported. It is concluded that the CIAV is highly prevalent among chicken flocks of Punjab state and presence of viral genome along with high antibody titer in apparently healthy birds indicates subclinical circulation of virus among most of the flocks. Moreover, the isolates obtained were closely related to other CIAV isolates found in India and the world.

Keywords: Chicken infectious anemia virus, ELISA, PCR, Virology, Molecular biology, Cell culture

Signature of Major Advisor **Signature of the Student**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	:	Per cent
µg	:	Microgram
µl	:	Microlitre
Ab	:	Antibody
ANOVA	:	Analysis of Variance
bp	:	Base pair
CIA	:	Chicken infectious anaemia
CIAV	:	Chicken infectious anaemia virus
CO_2	:	Carbon Dioxide
conc.	:	Concentration
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribo nucleic acid
dNTP	:	Deoxyribonucleoside tri-phosphates
DW	:	Distilled water
EDTA	:	Ethylene Diamine Tetraacetic acid
ELISA	:	Enzyme linked immunosorbent assay
FBS	:	Foetal Bovine Serum
g	:	Gram
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences
H_2SO_4	:	Sulphuric acid
hr(s)	:	Hour/s
ICTV	:	International Committee on Taxonomy of Viruses
iELISA	:	Indirect enzyme-linked immunosorbent assay
Ig	:	Immunoglobulin
kDa	:	Kilo Dalton
LB	:	Luria Bertani
M	:	Molar
mAb	:	Monoclonal antibody
Marker	:	100bp or 1kb DNA ladder
MDCC MSB1	:	Marek’s disease virus transformed lymphoblastoid cell
mg	:	Milligram
min	:	Minutes

ml	:	Milliliter
mM	:	Milli molar
ng	:	Nanogram
No.	:	Number
NSS	:	Normal Saline Solution
^{o}C	:	Degree Celsius
OD	:	Optical Density
ORF	:	Open reading frame
PBS	:	Phosphate buffer saline
PBS-T	:	Phosphate buffer saline-Tween 20
PCR	:	Polymerase chain reaction
PCV	:	Packed cell volume
pH	:	Hydrogen ion concentration
pmol	:	Pico mole
RBC	:	Red Blood Cells
RE	:	Restriction endonuclease
rpm	:	Revolutions per minute
s	:	Second
SDS	:	Sodium dodecyl sulphate
SPF	:	Specific pathogen free
Taq	:	*Thermus aquaticus*
TBE	:	Tris borate EDTA
TE	:	Tris EDTA buffer
TLC	:	Total leukocyte count
Tm	:	Melting temperature
U	:	Units
UV	:	Ultraviolet rays
VP	:	Viral protein
WBC	:	White blood cells

CHAPTER – I

INTRODUCTION

Poultry farming has emerged as one of the fastest growing segments of the livestock sector during the past few decades. Transformation from age-old backyard poultry faming into organised dynamic agricultural industry showed upward growth of poultry population in India. As per the 19th livestock census (2012), a 12.39% increase in poultry population with 729.2 million birds in numbers was recorded over a period of five years in India. Fowl shares 95% among total poultry population in India. India contributes 2.3% (5th rank) in the global meat production and 5% (3rd rank) in the world hen egg production. Among the various poultry producing Indian states, Punjab showed a phenomenal increase in poultry population (57.17%) during 2003-2012. The total poultry population in Punjab is 16.79 million in numbers which is mainly contributed by fowls (Livestock census 2012).

There are many advantages in poultry sector like reduced management responsibilities, almost fixed income, less risk of production, less operating capital etc. when compared with other livestock sectors. Among poultry, chicken is of great importance for meat and eggs. The growing human population, urbanization, potential for employment and subsidiary income generation, changing food habits (vegetarian to non-vegetarian) (Bennett *et al* 2018) are some of the driving forces for the recent growth in Indian poultry sector. Even if, there is an upward trend in poultry production, we could not achieve the ICMR recommendation (180 eggs and 10.8 kg poultry meat per person per annum) as the present per-capita availability of eggs is 54 and chicken meat consumption is 2.2 kg. Therefore, egg and chicken meat production have to be increased manifold to meet the high demand. Even though poultry farming is fast growing, it is also facing some challenges like susceptibility to emerging and re-emerging bacterial/ viral diseases. Some of the common avian pathogens are *E. coli*, *Salmonella spp*, Fowl Adenovirus, Egg Drop Syndrome-76 virus (EDS-76 virus), Infectious Bursal Disease virus (IBD virus) Marek's Disease Virus (MDV). Recently, Chicken infectious anemia virus (CIAV) has been reported among the chicken all over the world. Severe threat and economic losses due to CIAV in the chicken industry during the past few decades attracted researchers towards this minute virus.

Chicken Infectious Anemia Virus causes Chicken infectious anemia (CIA), a disease of young chickens characterized by aplastic anemia, generalized lymphoid atrophy and immunosuppression. It is highly contagious and can be transmitted both vertically and horizontally among birds. The infection occurs naturally when breeder flocks, just before or during egg production, with no previous exposure to the virus, become infected. Under these conditions, the progeny from infected parent develop the disease symptoms, including thymus atrophy, haemorrhages and anemia, from 10-14 days of age. Horizontal transmission is through the contaminated environment like litters, water, utensils etc. Clinical disease may not occur in chicks with high maternal antibody titer but do not prevent infection and transmission of the virus or immunosuppression (Balamurugan and Kataria 2006). The virus can infect chickens of all ages but only young chicks may develop clinical signs. It causes sever disease in young birds of 2-3 weeks of age. An age-related resistance was observed in adult birds and the virus can cause infection in adult birds but not disease. The economic importance of this disease is more in young chicks, it impairs the thymus causing immunosuppression which leads to major economic losses in broiler production with poor weight gain and heavy mortality. Both clinical and subclinical CIAV infections along with secondary bacterial/viral infections have a significant effect on commercial broiler/layer performance and profitability.

CIAV is one of the smallest viruses affecting chicken. It is having a single stranded circular DNA. The genome of CIAV carries three important partially overlapping open reading frame (ORF) namely VP1, VP2 and VP3 which codes three important viral proteins capsid protein, scaffold protein, apoptin respectively (Noteborn *et al* 1991, Zhang *et al* 2013). Capsid protein is the major viral structural protein, together with scaffold protein induce the production of neutralizing antibody (Noteborn 2004, Zhang *et al* 2013). Apoptin protein induce the apoptosis and apoptic bodies give characteristic doughnut-like structures of the infected cells. Among the three viral genes, highest nucleotide variability is present in VP1 gene.

Since its first occurrence in Japan, various studies untied much of the pathogenesis and epornithology of infection. Few studies in SPF birds were conducted to describe the pathogenesis and disease progress. It can be spread by either faecal oral route or vertically. The clinical form of disease usually occurs towards the end of

second week of life. The chicks became anorexic, depressed and 5-60% morbidity and mortality occur. Peak mortality occurs within 5-6days of onset of clinical signs and decrease by next 5-6 days. The lesions consist of focal ecchymotic haemorrhages on the musculature and skin, pale bone marrow with aplasia and reduced hematocrit values (<25%) (Rosenberger and Cloud 1998). Atrophy of the thymus along with depletion of bone marrow cells are considered diagnostic when associated with other signs and lesions. The birds may recover from the disease but growth may be stunted and are under higher risk for secondary bacterial/viral infection due to sever immunosuppression.

Diagnosis of CIA involve virus isolation (cell culture, embryonated chicken egg or SPF chicks), virus particle demonstration by electron microscopy, indirect immunofluorescence assay (IIF), serum neutralization test (SNT), Enzyme Linked Immunosorbent Assay (ELISA) and nucleic acid-based assays like polymerases chain reaction (PCR), restriction fragment length polymorphism (RFLP) and Real time PCR (RT-PCR). There is no specific treatment protocol and control measures can be formulated by proper vaccination of birds especially before they come into laying stage. The complete eradication of CIAV is very difficult as it is very hardy and ubiquitous in nature. Viral persistence in gonads of adult birds and vertical transmission further makes it difficult to control the infection in susceptible birds. Further, CIAV enhances the outburst of other bacterial, viral and fungal infection by immunosuppression. In India, the first report on CIAV was from Tamil Nadu (Venugopalan *et al* 1994). Since then, various studies involving virus isolation, molecular detection and serological studies have been reported from few regions or/states of India. Previous studies show high serological and antigenic prevalence of CIAV in the commercial as well as unorganised farms. Virus latency especially in the reproductive tracts of sero-negative flocks along with concurrent vaccine failure, and production loss also has been reported. As it is an emerging immunosuppressive disease of chicken, more detailed studies are needed to find out the current status of CIA in India. Although, few reports of molecular level studies for the prevalence of CIAV are available, no systematic analysis and genetic characterization of CIAV has been done in Punjab state to the best of our knowledge. A systematic analysis of CIAV circulating across the poultry intense Punjab state is necessary for taking effective control measures. Therefore, it is dire necessary to determine CIAV status in

the poultry farms of the Punjab state. Keeping these facts in mind, current study entitled “Isolation and Molecular characterization of Chicken Infectious Anemia Virus” was envisaged with the following objectives:

- To study the sero-prevalence of CIAV among chicken in Punjab state.
- To isolate CIAV from suspected birds/flocks and detect the isolated virus by molecular technique
- Molecular characterization of virus isolates by cloning and sequencing of the important viral genes or Whole genome sequencing

CHAPTER – II

REVIEW OF LITERATURE

3.1Chicken Infectious Anemia

Chicken infectious anemia (CIA), highly immunosuppressive disease of young chickens, is caused by Chicken Infectious Anemia Virus (CIAV). It emerged as one of the economically important disease of chicken during the last few decades that cause severe aplastic anemia and generalized atrophy of lymphoid organs in young chicks (Oluwayelu 2010). As it causes severe immunosuppression, CIAV plays an important role in many other secondary bacterial or viral infections leading to anemic syndrome in chicken. Hence, depending upon outward sign or symptoms, it has been commonly referred as *Hemorrhagic syndrome*, *Anemia dermatitis* or *Blue wing disease* (Todd 2000).

Among chicken, all the age groups are susceptible to infection with CIAV but clinical disease occurs only in chicks (1-3 weeks). Young chicks may get infection either vertically from infected layers or horizontally through contact with infected chicks, contaminated fomites, houses etc. The affected birds may show depression, more or less pale, and anemia is the only specific sign of CIAV infection (Bulow and Schat 1997). Thymic, splenic, bursal and bone marrow atrophy can be seen as gross lesions which may leads to immunosuppression. Further, secondary microbial infection may magnify the intensity of outcome of the disease. Due to severe infection and immunosuppression at younger age, mortality may range from 10% - 60% (Adair 2000, Todd 2000). Age related resistance were observed in adult birds since most of the birds are seroconverted either due to natural subclinical infection or due to vaccination of breeders just before laying period. Adult birds may get infection and recover from the disease but their growth performance (growth rate, feed conversion ratio, reduced production of egg and meat etc.) may get affected. Control of CIAV through vaccine is very challenging because of subclinical and immunosuppressive nature. Interaction of CIAV with other important immunosuppressive poultry viruses like MD, IBD, Avian leukosis and sarcoma virus (ALSV) etc. further enhance the persistence of virus among chicken.

3.2 History

The virus was first reported from Japan in 1974 (Yuasa *et al* 1979). Originally the virus was known as chicken anemia agent (CAA) but further morphological and biochemical studies changed its name to Chicken infectious anemia virus (CIAV). In their study, Yuasa and coworkers isolated a transmissible agent in experimentally infected specific pathogen free day-old chicks. At 14^{th} day of infection, 100% morbidity and 50% mortality were observed along with severe drop in hematological parameters like PCV, total erythrocyte count, total leucocyte count. Gross lesions like watery blood, yellow to pale bone marrow, atrophy of thymus and bursa of fabricius, swelling and discoloration of the liver, and sometimes hemorrhage throughout the body were observed. The first isolated virus was Gifu-1 strain from Japan (Yuasa *et al* 1979).

Later in 1983, Yuasa showed that the virus could produce cytopathic effect on certain lymphoblastoid chicken cell line (MDCC-MSB1) but not in conventional cell culture. This enabled extensive *in vitro* studies including serological and molecular biology studies on CIAV. Subsequently, it was reported from all the major poultry producing countries throughout the world including India (Gowthaman *et al* 2014).

3.3 Chicken infectious anemia virus (CIAV)

The smallest known virus of chicken was recently reclassified from the family *Circoviridae* to *Anelloviridae*, under Genus *Gyrovirus,* species *Chicken Anemia Virus* (ICTV 2017, Rosario *et al* 2017). The virions are nonenveloped with icosahedral symmetry and the diameter ranges from 19.1–26.5 nm (T=3 icosahedron with 32 morphological subunits) with spherical and/or hexagonal shape (Todd *et al* 1994) (Figure1A).

According to Bulow and Schat (1997), the virus usually shows type I (one central hollow surrounded by 6 hollows) and Type II (10 evenly placed protrusions) morphology as there is 5fold and 3fold symmetry of virus capsid (Figure1B). It has a buoyant density of 1.33-1.34 or 1.35-1.37 g/mL in cesium chloride (CsCl) and a sedimentation coefficient of 91S in isokinetic sucrose gradient. Noteborn *et al* (1994) demonstrated doughnut shaped inclusion bodies (aggregation of viral antigen) in infected MDCC cell line (Figure1C). The virions are very hardy in nature and resistant to harsh treatment with chemicals or heat.

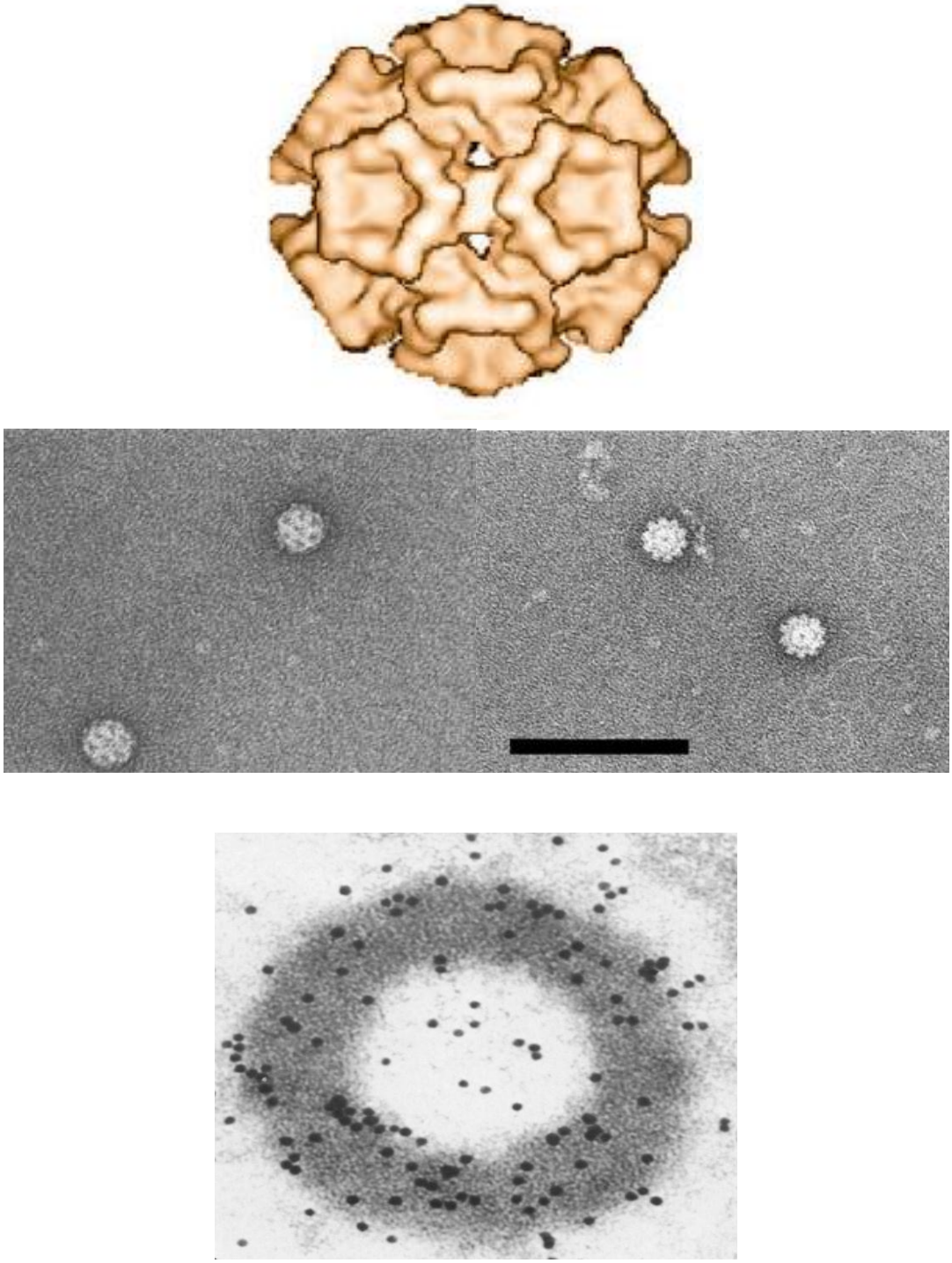


Figure.1 Chicken infectious anemia virus particle.

A) The capsid is formed from 12 pentagonal trumpet-shaped capsomeres. B) Negative-stained electron micrographs of Chicken infectious anemia virus shows different structural aspects of the capsids. Projection type I showing CIAV capsids that exhibit 6 stain-filled morphologic units that surround one central hole and Particle projection type II characterized by 10 peripheral protrusions (Bar = 100 nm) (Schat and Van Santen 2013). C) Aggregation of viral antigen in the nucleus of infected cells (McNulty *et al* 1990)

3.4 Chicken Infectious Anemia Virus Genome

Several molecular level studies have been done to characterize the genome of CIAV. The Genome consists of a single molecule of circular negative sense, ssDNA, which ranges from 1.7 to 2.3 kb in size (Noteborn 2004, Bulow and Schat 1997, Shulman and Davidson 2017) (Figure2). Three important overlapping (partially or completely) open reading frame (ORF) have been reported in the genome of CIAV (Kato *et al* 1995) with one promoter region, and one polyadenylation signal. ORF3 is located within ORF2 and ORF2 is partially overlaped with ORF1. The genome of CIAV is ambisense in nature as it can transcribe mRNA from both strands of replication intermediate during replication (Schat and Van Santen 2013).

Noteborn and Koch (1995) reported that all the ORFs and transcription elements (Promotor and polyadenylation signal site) are present in the plus strand of DNA. Various reports show the important position of open reading frame *i.e.* ORF1 with 1347 nucleotides (853-2199 nucleotide position), ORF2 with 648 nucleotides (380-1027 nt position) and ORF3 with 363 nucleotides (486-848 nt position) (Wani 2013). It is thought to replicate using the rolling circle replication (RCR) mechanism (ICTV 2017) with double stranded intermediate DNA. Cardona *et al* (2000) suggested that the double stranded replicative form may leads to the presence of latent episomal DNA in gonadal tissues.

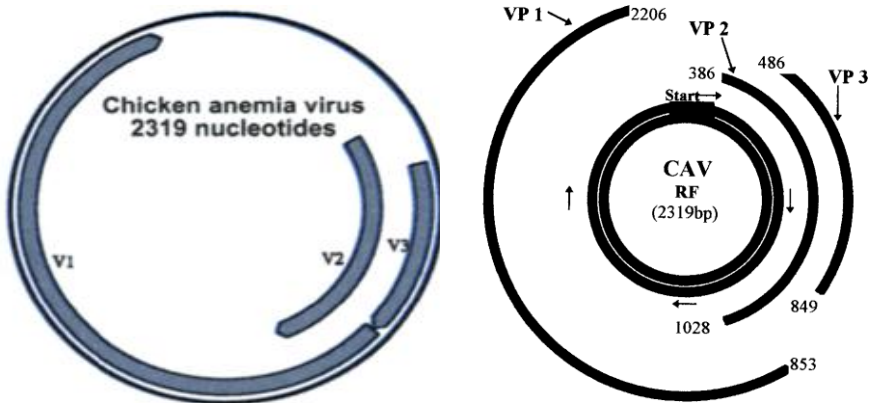


Figure 2: Genetic map of circular DNA of CIAV. A) a map representing CIAV genome with three important ORF. B) Replicative intermediate (RF-inner circle) of CIAV shows starting and ending position of VP1, VP2 and VP3 ORFs (Noteborn *et al* 1991)

Partial and complete genome sequence analysis of CIAV DNA have been reported from various part of the world (Meehan *et al* 1997, Santen *et al* 2001, Islam *et al* 2002, Simionatto *et al* 2006). These reports suggest that genome of CIAV is

highly conserved with minor difference in hypervariable region of VP1 gene (i.e. between 139 to 151 amino acid of VP1 protein). Large number of sequences data are available in the various databank which shows the very low variability in the nucleotide sequence of CIAV genome (Todd *et al* 1992, Renshaw *et al* 1996). Among the three viral genes, VP1 is the most variable gene responsible for neutralizing antibody production. Further, virus attenuation was observed after high number of passages in cell culture which is related to mutation in various nucleotide position especially in 139, 144 and 394 (Todd *et al* 1995, Yamaguchi *et al* 2001).

3.5 Important Viral Proteins

The coding region containing three partially overlapping ORF which encodes VP1 (51kDa/449AA), VP2 (26kDa/216AA) and VP3 (14kDa/121AA) proteins (ICTV 2011, Wani 2013). Among these, VP1 is structural protein and VP2 and VP3 are non-structural proteins. As the viral replication progress, the VP3 viral protein will appear as early protein that can be detected at 6 hours, while VP2 is present at 12hours post infection. The capsid protein VP1 is not detectable until 30 hours post infection. Various studies with VP2 mutant virus suggested that phosphatase activity of VP2 protein is important for virus replication but not necessary and it has a role in trafficking of VP3 protein from nucleus to cytoplasm. VP3 is essential for the virus replication cycle and apoptosis of infected cells. VP3 triggers loss of mitochondrial membrane potential and release of cytochrome c and apoptosis-inducing factor which triggers apoptosis via the mitochondrial pathway.

Noteborn *et al* (1991) studied on all the elements required for the virus replication cycle. VP1 is the only structural protein known to form viral capsid (coded by ORF1) and VP2 is the protein phosphatase (coded by ORF2) with serine/threonine and tyrosine protein phosphatase activities. Although, the full function of VP2 protein is unknown, it has been proposed that VP2 may act as a scaffold protein during virion assembly to facilitate the correct conformation of VP1.VP3 (coded by ORF3), is apoptin protein capable of causing apoptosis in infected cells especially in thymocytes and lymphoblastoid cells. The VP1 and VP2 proteins are present in the purified virion particles whereas VP3 will not be present.

Jeurissen *et al* (1992) observed that CIAV causes a complete depletion of the thymic cortex after *in vivo* infection and in cell lines after *in vitro* infection. They suggested that lymphoid depletion in CIAV infection is due to apoptin protein of

CIAV. Noteborn (1994) also supported the same finding that a single CIAV protein can induce apoptosis in chicken lymphoblastoid T and myeloid cells. Cells infected with CIAV showed granular distribution of VP3 protein within the nucleus which later forms aggregates. Aggregation of VP3 induce apoptotic process in the cells and the cellular DNA fragmented and condensed.

The studies on different recombinant viral protein to determine the development of antigenicity was done previously by various workers. Koch *et al* (1995) proposed that neutralizing antibodies were induced after inoculation of chickens with insect cells containing both VP1 and VP2, but not with cells containing only VP1 or VP2.

Cunningham *et al* (2001) reported that recombinant VP3 protein reacted specifically with anti-VP3 monoclonal antibodies (mAbs) and with serum from vaccinated chickens. But high non-specific reactions and false positive were observed in birds with different age group especially in older birds. They also suggested that VP3 protein is not suitable for use as antigen in immunodiagnostics as it is poorly immunogenic and low antibody concentrations are masked by non-specific reactions.

Noteborn (2004) reported that co-expression of VP1 and VP2 proteins is important for the production of neutralizing epitope. Recombinant VP1 or VP2 protein alone produce suboptimal neutralizing antibodies. Therefore, the viral proteins have important role in eliciting immune response in host

Trinh *et al* (2015) studied epitope mapping of VP1 protein with specific mAbs in escape mutants. The results suggest that two mAbs recognized the same epitope and these epitopes are topologically close in VP1 protein. Co-existence of these epitope found to be necessary for the reactivity of mAbs to epitope

3.6 Serotypes of CIAV

Based on antigenicity to viral proteins, different CIAV strains were reported from throughout the world. All strains belong to one serotype as there was no antigenic differences have been recognized in these strains (McNulty 1991, Bulov and Schat 1997). But epitope mapping and detailed studies on DNA suggested that immunodominant proteins may fold differently, resulting in variation of antigenicity development by various strains (Spackman *et al* 2002a, Spackman *et al* 2002b, Trinh *et al* 2015).

Spackman *et al* (2002a) reported a second serotype of CIAV named as CIAV-7. They compared the pathogenesis ofCIAV-7 that produces a clinical disease similar to CIAV but which is antigenically different. CIAV-7 has characteristics such as small size and resistance to heat, acid, and chloroform similar to CIAV.Also, CIAV-7–induced lesions were similar to the pathognomonic lesions induced by CIAV. Further investigation on CIAV-7 suggests that the lack of any antigenic cross reactivity using polyclonal chicken sera and lack of cross hybridization under low-stringency conditions, it can be a novel virus rather than a new serotype of CIAV (Schat and Santen 2013).

3.7 Chicken Infectious Anemia (CIA) – the disease

CIA is highly contagious acute disease, clinically characterized by severe anemia, generalized lymphoid atrophy and increased mortality in young chicks. Chicken of all age, sex, broilers, layers are susceptible to CIAV infection. Young chicks of 1-3 weeks of age get infection from the parent stock which got acute infection during their egg laying period (vertical transmission). Transmission of virus also occurs either through oral or faecal route (Horizontal transmission). Poultry farm management practise and CIAV contaminated vaccine also play important role in horizontal transmission of CIAV (Wani 2013).

There has been literature which reported the course of infection and disease progression in young and adult birds (Yuasa *et al* 1979, McNulty 1991, Adair 2000, Schat 2003, Balamurugan and Kataria 2006, Wani 2013). Clinical and subclinical forms of infection are observed in young and adult birds respectively. During egg production virus get transmitted to developing embryo and after hatching, virus target the hematopoietic cells (hemocytoblasts) in the bone marrow and thymocytes in the thymus. The peak virus load in thymus occurs during 10-14 days of infection. Therefore, unprotected young chicks (*i.e.* 1-3 weeks) without/ insufficient antibody are highly susceptible to CIAV. The maternal antibody may persist up to 20days and with declining maternal immunity, chicks of age group 20-30 days are also highly prone to infection and disease. In adult birds, clinical form of disease is not occurring but sub-clinically virus circulate among birds without any outward signs or symptoms (Miller *et al* 2004, Schat and Van Santen 2013).

Smyth *et al* (2006) studied the mechanism of disease progression in experimentally infected older birds of different age group. Their study reported that

horizontally acquired CIAV infection has subclinical effects on chickens, and that may be due to immunosuppression caused by viral damage to the thymus.

3.8 Clinical signs

Acute form of disease was reported in both broilers and replacement pullets with an incubation period of 10-14 days (McNulty1991). Affected birds show depression, anorexia, pale comb and wattle, ruffled feathers and stunted growth, weight loss and death within one week of infection. Peak mortality occurs within 5-6 days of onset of clinical sign. Focal skin lesions have been observed on the wings, head, on the sides of the thorax and abdomen, on the thighs, legs and feet and ecchymotic haemorrhages on the skin (McNulty1991). The only specific sign observable is anemia which become severe at second week of infection (Bulov and Schat 1997). CIA associated haemorrhages can be due to decreased clotting time and thrombocytopenia. Therefore, the skin become bluish and breaking which may attract secondary bacterial infection leading to gangrenous dermatitis. The death of the affected birds may occur between 12-28 days of post infection. Gross lesion consists of severe bone marrow aplasia and pancytopenia, with reduction in haematocrit values ranging between 6 - 27% (PCV < 25%) (Balamurugan and Kataria 2006).

Wani *et al* (2013) described various gross lesions like pale bone marrow, discoloured or swollen liver, atrophy of the thymus, bursa of fabricius, areas of congestion and haemorrhages in visceral organs due to CIAV infection.

3.9 ***Haematological profile***

In birds, generally hematocrit values greater than 27% are considered normal, but values may depend on various factors (Jaffe 1960). Gross appearance of blood in affected chicks were more or less watery with increased clotting time. Hematocrit values begin to drop below 27% at 8–10days after infection, are mostly in the range of 10–20% at 14–20days, and may even drop to 6% in moribund birds (Goryo 1989, Schat and van Santen 2013). Severe anemia in affected chicks is the pathognomonic sign of infection with CIAV. Infection of hemocytoblasts results in decreased numbers of erythrocytes, white blood cells, and thrombocytes which leads to pancytopenia (Taniguchi *et al* 1983, Pope 1991, Adair2000). Convalescing chicks may show increase in hematocrit values after 16–21 days and 29–35% recovery observed after 28-32 days of post infection (Schat and van Santen 2013)

Krishan *et al* (2015b) reported a significant decrease in Hb, PCV and TEC in the chicks experimentally infected with CIAV from 7^{th} to 21^{st}day of post infection. They observed that a severe decrease in Hb concentration from 116-121 g/Lto 52g/L in CIAV infected chicks on 21^{st}day.

Wani *et al* (2015) analysed hematological parameters during experimental subclinical infection of Chicks with CIAV. Their results showed significant decline in PCV, TLC and PLC after 15 days post infection but there were no clinical signs of CIA in birds under study.

Narayani and Ghosh (2018) studied with a recent field isolate of CIAV which produced consistent clinical signs of CIA and low haematocrit values in experimentally infected day-old chicks. They observed a significant decrease in haemoglobin, PCV, TLC and Lymphocyte values.

3.10 ***Morbidity and Mortality***

The outcome of the CIAV infection is dependent on the various factors like vertical or horizontal transmission, virulence of the strain, dose and route of the infecting CIAV and host factors. Parenteral rout of inoculation of the virus is more effective than oral, nasal or ocular route. Morbidity is usually occurring between 20-60% (McNulty1991). Maternal antibody may protect the chicks but may not be effective if the birds are immunocompromised. Also, age-related resistance closely related to the ability of bird to produce antibodies due to an active infection. Concurrent infection with other immunosuppressive disease like IBDV, MDV, REV may enhance the morbidity and mortality

Goryo *et al* (1985) reported that the strain of CIAV TK-5803 were highly virulent than the isolates studied by Yuasa and Imai (Bulov and Schat 1997). A 100% morbidity was reported by Wani *et al* (2014)

Engstrom and Luthman (1984) were recorded 60% mortality in experimentally infected birds. But Wani *et al* (2014) observed a 10% mortality as it is usual in CIAV infection (5-10% mortality).

3.11 Pathology and pathogenesis

The pathogenesis of CIAV infection during natural or experimental exposure were reviewed by various workers. The Virus require actively dividing cells in lymphoid organs for its replication. Within 3–4 days of post infection, a considerable

reduction in stem cells were observed due to apoptosis of the cells induced by VP3 protein of CIAV.

Goryo *et al* (1989) observed that primary target cells to be hematopoietic precursor cells (hemocytoblasts) in thymus cortex and thymic precursor cells (lymphoblasts) in the bone marrow in early stage of infection. It is observed that CIAV have specific tropism towards the lymphoid cells (precursor T cells in Thymus, bone marrow and spleen)

McNulty (1991) reported that replication of virus causes severe destruction and apoptosis of the cells in the lymphoid organs which leads to aplastic anemia, thrombocytopenia, leukopenia and thymus atrophy. Because of this, there will be impairment in the T-lymphocytes and macrophage activity which leads to severe immunosuppression in young chicks (McConnell *et al* 1993). Also, Depletion of lymphoid organs and immunosuppression increase the susceptibility to secondary microbial infections (bacterial, fungal and viral) and depression of vaccinal immunity. Similar findings were observed by Smyth *et al* (1993).

Adair (2000) well described the immunopathogenesis of CIAV infection in young birds of 2-3 weeks of age. once virus entered into the host, it targets the erythroid and lymphoid progenitor cells in the bone marrow and thymus respectively. Destruction of erythroid progenitor cause severe anemia and depletion of granulocytes and thrombocytes. Destruction of lymphoid progenitor (precursor T cells) causes depletion of mature cytotoxic and helper T cells.

Smyth *et al* (2006) suggest that CIAV is capable of infecting thymocytes of older birds (>6 weeks) and that it is associated with lymphocyte depletion. There was only limited evidence of viral replication in the other tissues examined

Along with initiation of antibody production, repopulation of lymphocytes, proerythroblast and promyelocytes have been observed. This results in complete recovery of the birds by 32-36 days. However, maternal antibody and age-related resistance may over power by coinfection with other immunosuppressive avian pathogens. Samy and Naguib (2018) have discussed the coinfection of various avian pathogen and suggest that better understanding of interaction of these agents can lead to improved intervention strategies to control the virus spread.

3.12 Attenuation of the CIAV

When the virus is passaged a number of times in a host system it changes its genetic makeup to adapt to the new environment. That kind of genetic changes causes attenuation of the virus. A number of reports suggested the difference in the development of pathogenicity by attenuated virus.

Bülow and Fuchs (1986) studied pathological aspect of cell culture attenuated Gifu-1 and Cux-1 strains of CIAV. The pathogenicity was found decreased after 12^{th} number of passages in cell culture and it was decreasing with increasing the passage number. But virus strains did not lose its pathogenicity completely even after 100 number of passages. However, Yuasa (1983) and Goryo *et al* (1987) found no decrease in pathogenicity of other CIAV isolates after 19-40 passages.

Todd *et al* (1995) found that Cux-1 became substantially less pathogenic after 173 passages in MSB1 cells.

Chowdhury *et al* (2003) observed that the repeated passages of the isolates in cell culture until passage 60 (P60) and passage 123 produced low pathogenic viruses that showed a significantly reduced level of pathogenicity in SPF chickens compared to the low passage isolates. Sequence comparison indicated that nucleotide changes in the coding region of the P60 passage isolates were thought to contribute to virus attenuation.

3.13 Diagnosis of CIA

Diagnosis of CIA is primarily based on clinical profiles, history of flock, signs and symptoms, haematological changes, gross pathological lesions (in autopsy of affected birds) along with the knowledge of its differentiation from other avian pathogens like MD, IBD, AL etc. Further diagnosis can be made by various methods for detection of antigen, antibody or molecular level detection of viral genome (Yuasa and Imai 1986, McNulty *et al* 1988). Confirmatory diagnosis can be done by isolation and identification of the causative agent in cell culture, SPF day old chick or ECE (Yuasa 1983, McNulty *et al* 1989, Bulow and Schat 1997) or demonstration of virus by Electron microscopy. Virus isolation in cell culture is more convenient but less sensitive than inoculation in chicks. Other methods like virus detection by immunofluorescent technique (McNulty *et al* 1988), serological test like indirect immunofluorescent test (IIFT), virus neutralization (VN), enzyme linked immuno-

sorbent assay (ELISA), and immunoperoxidase (IP) tests (Saini and Dandapat 2009) and nucleic acid amplification methods like polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) (Subramanyam *et al* 2010, Krishan *et al* 2015) can be done as supporting test.

3.14 Isolation and identification of CIAV

Isolation of the CIAV can be achieved by inoculation of the suspected material in embryonated eggs (SPF egg), cell line (MDCC-MSB1 cells) and day-old SPF chicks which is considered as gold standard method. CIAV isolation can be achieved in one or two passages in chicks or four to five passage in cell culture or ECE (Yuasa 1983, McNulty *et al* 1989). All tissues of infected chicken *vis* thymus, liver, bone marrow, spleen, bursa, lung, muscle, Feathers, blood (buffy coat) and rectal contents can be used for isolation of the virus (Yuasa *et al* 1979, McNulty 1991).

3.14.1Cell culture Method

Isolation of CIAV in various cell line were attempted but it was found that lymphoblastoid cell lines were most suitable for virus growth and multiplication. Yuasa (1983) Propagated chicken anemia agent in a cell line (MDCC-MSBI) derived from Marek's disease transformed lymphoblastoid cells and infectivity titration of the strain Gifu-I was done. Initial changes in infected cells are not much appreciable but after passaging for 5-6-time visible damages can be well identified. These changes are suggestive CIAV growth. Cytopathic effect (CPE) produced by the virus can be characterized by observing enlarged, swollen and misshapen cells, cell degeneration and lysis, alkaline red medium, inhibition of cell growth and ultimately the inability to subculture. Further, inoculation of clinical specimen into the cell culture or SPF birds enables biologic amplification of virus particles to levels where they can be detected by electron microscopy (EM). Similar findings were observed by various other workers. Confirmation of the isolated virus can be done either by IIFT or demonstration of CIAV by electron microscopy (McNulty *et al* 1988, McNulty *et al* 1990).

Rosenberger and Cloud (1989a) isolated the virus in one day old SPF chicks by I/M or I/P route. The agent induced a severe bone marrow aplasia, thymic atrophy, multiple subcutaneous and intramuscular haemorrhages, and anemia, as evidenced by reduced haematocrits. Similar studies were also conducted by various other researchers (McNulty *et al* 1989, Lucio *et al* 1990, Brentano *et al* 1991).

Renshaw *et al* (1996) suggested that field isolates of CIAV can vary significantly in their ability to replicate in MDCC-MSB1 cells and different sublines of MDCC-MSB1 cells also may vary in their susceptibility to CIAV. Genetic make up of the virus may change after a number of passages in cell culture and that affect the virus growth and spread.

3.15 Demonstration of CIAV by Electron Microscopy (EM)

Identification of the isolated virus by electron microscopy were critical to initial recognition of the etiologic agent, which can be further analyzed by additional confirmatory laboratory diagnostic assays. The classical technique like cell culture and EM studies compliment the advances in molecular technique and allows researchers an unbiased recognition of causative agents in the clinical specimen. Various works has been reported to characterize the morphology of the virus including CIAV and ultrastructural changes in virus infected cell line.

Goryo *et al* (1987) reported the biological characterization and electron microscopic features of CIAV isolated in MDCC-MSB1 cell line.

McNulty *et al* (1990) demonstrated CIAV isolated in MDCC MSB1 cells. the characteristic morphology of CIAV particles negatively stained with uranyl acetate were found to be 26.5 nm in diameter. Also, they demonstrated the capsid with 32 structural subunits arranged as in a class P=3 icosahedron with a triangulation number of 3. Similar studies were also reported by Todd *et al*(1990).

Noteborn *et al* (1994) demonstrated characteristic doughnut like inclusions in infected MDCC MSB1 cells. Presence of virus in tissues of SPF chicks inoculated with CIAV were also demonstrated by transmission electron microscopy.

Crowther *et al* (2003) compared structures of three viruses namely Porcine circovirus (PCV), Beak and feather disease virus (BFDV) and Chicken infectious anemia virus. They have analysed the detailed structures and morphological relationships between the three viruses. The three-dimensional maps show PCV-2 and BFDV to have a diameter of about 20.5 nm, while the diameter of CIAV is about 25 nm.

After initial recognition, the findings can be confirmed by other techniques like serologic testing, immunohistochemical (IHC) test and indirect fluorescence antibody (IFA) assays. Further, molecular characterization of the virus can be done by

various methods like PCR, RFLP, RT-PCR, etc. which facilitate the differentiation of the virus into species and strain (Goldsmith *et al* 2013).

3.16 Demonstration of CIAV by Immunological Assays

Immunoassays like immunofluorescence, Immunoperoxidase were used for demonstrating antigen in virus infected cell line or tissue impression smears. Dhama (2002) and Wani *et al* (2014) in separate studies demonstrated virus-specific antigen in infected MSB1 cells and cryostat-sections of infected tissues by indirect immunofluorescence staining. Fluorescent staining of small, irregularly shaped granules in the nucleus of enlarged cells is observed in positive cases of specific immunofluorescent antigens. Employing monoclonal or polyclonal antibodies to apoptin protein produced immunofluorescence which can be characterized by doughnut shaped granular apoptic bodies.

Immunoperoxidase (IP) staining technique has also been used for detection of CIAV antigens efficiently in tissue sections. Smyth *et al* (1993) showed infected cells with specific deposition of a brown staining particles in the nucleus and cell membrane. Thymus and bone marrow are the tissues of choice for immunostaining.

3.17 Serological diagnosis

Serological diagnosis of CIA can be done by various methods like ELISA, IIFT, VNT etc. Detection of anti-CIAV antibody in the serum of the birds can be done by these methods. Serological assays are efficient tool for monitoring the outcome of vaccination, CIAV status in SPF chicks and ECE used for vaccine production. In that way, the risk of CIAV contamination of avian vaccines with CIAV can be avoided. Among the ELISA, IIFT and VNT, the later one is highly sensitive but it takes 5 weeks with nine to ten sub culturing of MDCC MSB1 cell line.

Otaki *et al* (1991) compared the sensitivity of ELISA, IFA, and VNT to detect anti-CIAV antibody and they observed that VN test was more sensitive than the other two assays.

Todd *et al* (1999) have developed an ELISA. They claimed that blocking ELISA based on the MAb 2A9 performs better than the IIFT, IIPT, indirect ELISA and is well suited for serological diagnosis of CIAV in SPF and commercial chicken flocks. On the basis of speed, cost and being more suitable for testing large numbers of serum samples, ELISA should be considered as the test of choice for investigating the epidemiology of CIA infection in poultry

Oluwayelu (2010) suggested that anti-CIAV antibody can be detected by IIFT, IIPT and VNT. But these tests have been superseded by ELISA. Various work has been done to develop ELISA which can be efficiently used for monitoring CIAV. Even though various commercial ELISA kits are available, all of them have to be imported, making the cost too high for large scale screening of antibodies to CIAV in chickens.

Chansiripornchai *et al* (2013) standardized and developed indirect ELISA to detect antibodies against chicken infectious anemia virus. Relative sensitivity, specificity and accuracy of in-house ELISA were 93%, 78% and 86%, respectively. Similarly, Wani (2013) standardized a recombinant protein (VP1) based indirect ELISA with a specificity, sensitivity and accuracy of 95.23%, 70.73% and 79.03 %.

3.18 Nucleic acid-based diagnosis of CIAV

Virus isolation and serological assays were normally used to diagnose CIAV infection but such techniques have some limitation. These tests are not suitable for use on decomposed tissue samples. Nucleic acid-based techniques are more sensitive, specific and rapid. The techniques like PCR, RT-PCR, RFLP, Nucleic Acid Hybridization were used by various researchers to detect and identify the CIAV genome.

Kataria *et al* (1999) confirmed the presence of CIAV in tissues of birds showing clinical signs of CIA by employing PCR.

Dhama (2002) reported variation among six isolates of CIAV recovered from different geographical regions of the country by RFLP analysis of PCR amplified products.

Caterina *et al* (2004) developed a multiplex PCR (mPCR)for detection of important avian DNA viruses *vis* avian adenovirus, avian reovirus, infectious bursal disease virus, and chicken anemia virus. These viruses are highly pathogenic, immunosuppressive and are transmitted rapidly among birds. The developed mPCR can use one faecal cloacal swab sample for testing of all four viruses which allows a quick, sensitive, and specific diagnosis of the disease in a flock.

Natesan *et al* (2006) studied virus isolates from different geographical regions of India. A complete coding sequence of VP2, VP3 gene and partial sequence of VP1 gene were amplified by PCR.

Brentano *et al* (2005) carried out nested PCR on birds experimentally inoculated with vaccine strain of CIAV. They found out the persistence of CIAV in the gonads and embryos of commercial broiler breeder chickens even after the seroconversion.

Praveen *et al* (2008) reported the PCR detection and isolation CIAV from affected poultry flocks of Gujarat and Andhra Pradesh States, India. They could successfully isolate CIAV in MDCC-MSB1 cell cultures from PCR positive field sample.

Mohamed (2010) investigated the presence of chicken anemia virus in broiler chickens with a history of depression, incidence other diseases, vaccination failure and mortalities (4-21%). A VP1 based PCR analysis showed 26.6% (44/165 birds) prevalence of CIAV in Egypt

Nineteen flocks (42.2%) of breeder farms and 11 cases of commercial chicken farms were found positive for CIAV by using PCR analysis (Kim *et al* 2010). Phylogenetic analysis of the VP1 gene, including a hypervariable region of the CIAV genome, indicated that Korean CIAV strains were separated into different groups. Korean CIAV groups showed similar amino acid pattern of I75, L97, I125, Q139, Q144, V157, and A413.

Wani *et al* (2013) studied molecular epidemiology of CIAV in India. VP1 VP2 and VP3 gene base PCR were used to amplify CIAV genome. The results show that a wide distribution of CIAV with 73.3% prevalence in commercial poultry farms.

Nayabian and Mardani (2013) amplified CIAV DNA from liver tissues of apparently healthy broiler chicken from slaughterhouse using PCR method. A 1.4 kb fragment comprising of complete VP1 gene was amplified by PCR. Also, the VP1genes of four CIAV isolates showed different RFLP patterns indicating genetic variation among the CIAV circulating in Iran.

Vagnozzi *et al* (2018) proposed that the best time to detect the virus in tissue is 7th day of post infection (DPI) using qPCR. CIAV remained detectable in tissues like thymus and spleen from 7 DPI through 21 DPI despite the presence of ELISA antibodies which were detectable from 14 DPI. Feather pulp was the tissue of choice for non-invasive CIAV detection.

Nucleic Acid Hybridization of CIAV-DNA in suspected tissue or blood can be done using southern blot analysis or dot-blot hybridization assay employing 32P or digoxigenin/biotin labelled DNA probes. The assay may be very useful in molecular pathogenesis and epidemiology and detecting subclinical infections (Noteborn *et al* 1991).

Restriction Fragment Length Polymorphism is one of the easiest nucleic acid-based technique that shows variation in homologous DNA sequences. Noteborn *et al* (1992b) compared the DNA genome of various CIAV isolates to that of cloned CIAV-DNA by RE digestion which shows minor differences in few CIAV field isolates.

3.19 Economic impact, Prevention and control

The negative impact of CIA on economy is mainly due to huge mortality in chicks, the cost of treatment to prevent or control secondary microbial infection and poor performance of recovered bird. Mcnulty (1991) reported that a 17-19% reduction in income per 1000 bird was observed in flocks having vertically transmitted CIAV. An average weight of 3.3% less and an average mortality of 2% more was observed in affected birds when compared to unaffected flocks. It causes severe economic threat to the poultry industry especially to broiler producers and SPF chick and egg producers.

Davidson *et al* (2004) reported weight loss and increased feed conversion ratio in CIAV infected birds. Various studies on economic impact of CIAV showed production loss, decreased weight gain, suboptimal feed conversion ratio. Importance of CIAV in SPF chick and egg is depends on legislation for vaccine production in various countries. European Union requires the absence of CIAV from eggs used for the production of all poultry vaccines and in Australia, Europe, and the United States requires the absence of CIAV in SPF eggs used for human vaccines like measles and mumps (Schat and van Santen 2013).

For the prevention and control, special attention needs to be given in management and hygiene procedures to prevent immunosuppression and further occurrence of disease. Due to vertical transmission, viral persistence, and hardy nature of the virus, it is practically impossible to eradicate CIAV from the poultry premises. Vaccination of young breeder flocks is the current strategy of prevention of anemia and disease in young chicks. Maternal derived antibody may protect the young birds

from natural infection. Vaccination should be performed at about 9–15 weeks of age, but never later than 3–4 weeks before the first collection of hatching eggs to avoid the hazard of vaccine virus spread through the eggs. But various report on molecular study on CIAV showed that recombinant stains of CIAV is circulating among chicken flocks. Most of these isolates were found to be highly pathogenic. Therefore, along with preventive measures time to time monitoring of CIAV among chicken flocks is very important for the control of CIA (Schat and van Santen 2013, AboElkhair *et al* 2014, Ou *et al* 2018)

3.20 Epidemiology (National and international status)

CIAV has been reported from almost all the continents including Asia. Since last four decades, it was emerged as an economically important avian pathogen. A number of factors influence the occurrence of infection and disease among birds. Age, dose and route of infection, mode of transmission (vertical and horizontal), co-infection with other avian pathogen and presence of neutralizing antibodies are some of the important factor for the development disease (Bulow and Schat 1997, Oluwayelu 2010). Maternal antibody and vertical transmission of virus through the egg are crucial for the epidemiological surveillance. Various Serological and molecular survey for assessing CIAV antibody and genome were carried out globally.

3.20.1World scenario

Ledesma *et al* (2001) conducted serological survey in Mexico which suggest that the virus is widespread in the Republic of Mexico. The results showed seroconversion in both layers and broilers from all the states under the study (All birds were unvaccinated) which indicated endemicity of the CIA.

Owoade *et al* (2004) found that 55% of 20 chicken flocks were found positive for CIAV antibodies in southwestern Nigeria. Out of seven farms screened 86.6% were found positive for CIAV antibodies. Another study by Emikpe *et al* (2005) in Nigerian indigenous birds reported that 88.9% of apparently healthy birds were found seroconverted to CIAV.

Toro *et al* (2006) conducted a retrospective, serological survey in United States which showed that the chickens could have been infected long before its first isolation in 1979.

Mohammadreza (2010) suggested that lower growth performance and inadequate response to vaccination observed frequently in broiler chicken farms of Shahrekord region can be due to CIAV infection

Biđin *et al* (2010) conducted serological study in unvaccinated broilers and broiler breeders in Croatia and they suggest that high presence of CIAV-antibody in broiler breeders are protective in nature.

Snoeck *et al* (2012) reported that an overall flock seroprevalence of 36.7% was observed in Central African Republic and Cameroon during 2006-2010. A trend of higher to lower sero-prevalence was reported from this geographical area. The cloacal swabs of seropositive chickens (76.9%) also found positive for CIAV DNA.

Eltahir *et al* (2011) carried out molecular epidemiological study and concluded that out of 460 spleen samples screened by PCR, 47 (10.22%) were found positive for CIAV specific gene. Analysis of CIAV genome reveals that virus circulating among commercial poultry are highly pathogenic.

Nayabian and Mardani (2013) used PCR-RFLP and nucleotide sequencing procedures to detect and differentiate CIAV nucleic acid in tissue extracts from infected birds. Experimental infection of birds with CIAV and other important pathogens like Infectious Bursal Disease Virus or Mareks Disease Virus resulted in high morbidity and mortality (Rosenberger and Claud1989b, Miles *et al* 2001).

Simeonov *et al* (2014) from Bulgeria reported 88 to 100% seroprevalence in backyard poultry and significant association between the seropositivity and age of birds under study was found.

Ou *et al* (2018) conducted molecular surveillance of CIAV genome and 52.55% were found positive for CIAV. The characterization of the detected virus revealed an inter-genotypic recombination.

There is no specific treatment exists and needs time to time surveillance of CIAV among the flocks for proper containment CIA. Live attenuated vaccines are available but vertical transfer, viral persistence and reversion of viral virulence is the important problems by using these vaccines. Therefore, new generation vaccines are explored (Sawant *et al* 2015). Molecular studies on VP1, VP2 and VP3 gene of CIAV has been reported for production of recombinant protein and development of sero-diagnostic assay (Iwata *et al* 1997, Lee *et al* 2008).

3.20.2 Indian scenario

In India, the disease was first reported from Tamil Nadu (Venugopalan *et al* 1994). Venugopalan *et al* (1994) demonstrated CIAV while investigating an outbreak of multifactorial etiology in young chicks of poultry from Namakkal, Tamil Nadu.

Dhama *et al* (2002) reported that PCR analysis and transmission studies conducted in different parts of the India shows the emerging tendency of CIA in India.

Bhatt *et al* (2011) reported high prevalence (86.88%) of CIAV antibodies in Northern India while Wani *et al* (2013) reported a prevalence rate of 73.3% by PCR detection of CIAV from clinical samples collected from different states of the country.

Gowthaman *et al* (2014) reported the subclinical nature of the virus. They report that 16 Indian isolates shared 98.8 – 100 % homology among them and was closely related to Brazil, Malaysia and Bangladesh.

Krishan *et al* (2015) conducted molecular surveillance of the CIAV, IBD, IBH, fowl pox, gout, CRD and MD in suspected poultry by various molecular epidemiological (PCR, RFLP etc.) study and found that high prevalence and co-infection of CIAV along with MD, IBD and IBH.

3.21 Genome analysis of CIAV

CIA one of the economically important disease with worldwide distribution. Some part of the world, it is highly prevalent and outbreaks was reported from various part of the world. The genome of chicken anemia virus has been analysed by various researchers. Isolates from the various region of the world show limited sequence variability. All isolates, perhaps, belongs to single serotype but different genotypes. The whole genome analysis of various isolates shows that there could be intergenotypic recombination event that may decide the virulence of virus.

Renshaw *et al* (1996) reported that among the three viral genes, VP1 is having the hypervariable region and the protein encoded by VP1 and VP2 gene may elicit neutralizing antibody. Therefore, time to time monitoring of CIAV circulating in the field is necessary to ascertain the genotypic and pathogenic variant present in the field.

Islam *et al* (2002) from Bangladesh studied sequence analysis of CIAV genome. A full-length PCR amplicon of the genome designated as BD-3 CIAV, was cloned and sequenced. The deduced amino acid sequences of VP1 showed different genetic groups among CIAV strains. Four signatory amino acids in VP1 observed are 75 (I/T), 97(L), 139(Q) and 144(Q), out of which the latter two are located in a small hydrophilic peak.

Natesan *et al* (2006) from India characterized four CIAV isolates from various regions of the country. In CIAV genome, a region consisting of 1766 bases containing the complete coding region of VP2 and VP3 proteins, and partial coding region of VP1 protein were sequenced. Their results indicated that Indian isolates were genetically evolved from different parts of the world. Indian isolate namely CIAV-A was found closely related to European Cux-1 strain, CIAV-B and -P were closely related to Bangladesh BD-3 strain and CIAV-E was closely related to Australian 704 strain. All Indian isolates had amino acid Q (glutamine) at position 394 in VP1 (a major determinant of pathogenicity). Further, pathogenicity study of all Indian isolates except CIAV-B were able to produce clinical disease and immunosuppression in young chicks whereas the isolate CIAV-B induced only immunosuppression.

Kim *et al* (2010) from South Korea have analysed hypervariable region of the CIAV genome. The results of both nucleotide and amino acid sequence comparison indicated that Korean strains were separated into different groups. Korean CIAV groups II and IIIa showed similar amino acid pattern of I75, L97, I125, Q139, Q144, V157, and group A413.Another group IIIb showed an L125, S287, G370, and S413 amino acid pattern. Based on sequence analysis, south Korean isolates are showing 98.9%nucleotide homology of VP1 gene with vaccine stain. The only variations from vaccine strain were 2 amino acid at positions 75 and 287 and that may be due to vaccine strain mutation leading to cluster formation. Vaccination of chicken breeder flocks with live attenuated vaccine has been granted by the government to control the continuous economic losses caused by CIAV in South Korea. Even though, vaccinated breeder flocks showed presence of mutated CIAV.

AboElkhair *et al* (2014) from Egypt conducted molecular study on Chicken anemia virus circulating in chicken flocks. They could amplify 675 bp nucleotide of partial coding region in VP1 protein. Analysis of nucleotide sequence and deduced amino acid showed that CIAV detected was not related to vaccine strain but can be a

field strain. The phylogenetic analysis from the deduced amino acid showed grouping of virus in to one group with other reference isolates with amino acid profile of I-75, L-97, Q-139, and Q-144 in VP1 protein.

Olszewska-Tomczyk *et al* (2016) from Poland studied Phylogenetic analysis of 16 CIAV isolates circulating in the country. Sequence analysis of CIAV isolates with various globally reported sequences revealed a low genetic variation at the nucleotide level. The nucleotide changes observed were silent mutations which indicated by a low diversity of amino acid sequences. They have also reported the vaccine like virus is circulating with other field strains which may lead to recombination events resulting in the emergence of new CIAV genotypes.

Li *et al* (2017) from China isolated Chicken Infectious anemia virus from broiler chicken. They could identify 22 CIAV strain from China. Full length genome sequences showed that all available CIAV could be classified into eight lineages. Putative genetic recombination events were also detected in the genomes of newly isolated CIAVs.

Ou *et al* (2018) characterised field isolates of CIAV circulating in Taiwan. Even though, vaccination of breeders was practised in with CIA to protect the flocks with maternal antibody, there was incidence of CIA among birds. Phylogenetic analysis of the VP1 gene revealed that Taiwanese isolates belonged to different genotypes. Taiwanese isolates showed close relation with vaccine strains (26P4 or Del-Ros) used in the country. They suggest that inter-genotypic recombination may be occurred between viruses of different genotypes.

Erfan *et al* (2018) conducted molecular study on CIAV isolated from Egypt and genotype was characterized. Full genome sequencing of 18 CIAV positive samples were grouped into four distinct genotypes. Deduced amino acid of VP1 protein have Q139 and Q144 amino acid substitutions among Egyptian field strains. Recombination events was evident in four Egyptian CIAVs which grouped in cluster A. which are known to be important in virus replication and spread.

CHAPTER – III

MATERIALS AND METHODS

The present study was conducted in the Virology laboratory of Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab.

3.1. Sample collection

3.1.1Blood samples for serological and molecular study

Blood samples were collected from either unvaccinated apparently healthy or CIAV suspected/ clinically ill birds from organized farms located in the various regions of Punjab state (Table 1). Obtained permission from IAEC (vide Memo No: IAEC/2016/310-335) for collection of blood samples from live birds. Samples were collected randomly from various farms during the period between 2015 to 2018.

Table 1: Details of blood samples collected from chicken

S. No:	Geographical area	Number of Organized farms	Number of birds
1.	Moga	5	9
2.	Ludhiana	4	38
3.	Sangrur	2	6
4.	Fazilka	4	14
5.	Tarantaran	1	5
6.	Hoshiarpur	2	6
7.	Patiala	2	5
8.	Pathankot	2	4
9.	Mansa	1	5
10.	Malerkotla	1	7
11.	Jalandhar	2	15
12.	Ahmedgarh	1	6
	Total	**27**	**120**

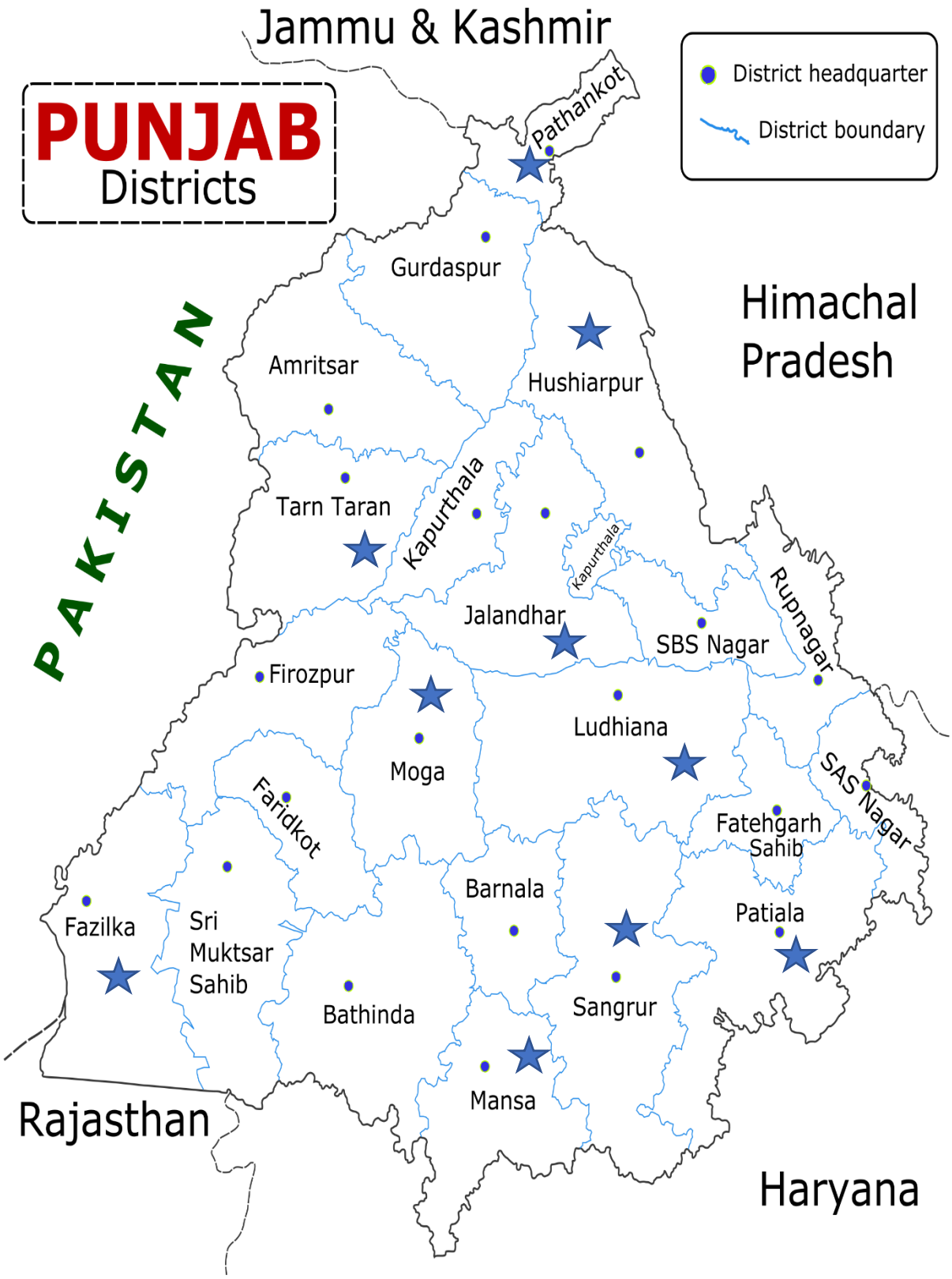


Figure 3: Districts of Punjab included in the present study. Presence of CIAV could be detected from Central, Eastern and southern part of Punjab.

3.1.1.1 Processing of blood samples for haematological studies

One ml of blood was collected from the wing veins of birds using sterile scalp vein set of 24G size and directly transferred to blood collection vials treated with ethylene di-amine tetra acetate (EDTA) as anticoagulant. Immediately after collection, the whole blood was used to analyses various haematological parameters like packed cell volume (PCV), haemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC) *etc.* Whole blood was stored at -80°C for further use.

3.1.1.2 Recording of haematological parameters

Packed Cell Volume (PCV): Micro-capillary method described by Al-Hassani (2011) was followed to measure PCV.

1. 2/3rd of the micro-haematocrit capillary tubes were filled with uncoagulated blood
2. Sealed the end with artificial mud
3. Tubes were placed on micro-haematocrit centrifuge and centrifuged at 10,000rpm for 5minutes
4. Readings were recorded with haematocrit ruler

Haemoglobin (Hb): Drabkin'smethod was used to estimate haemoglobin from the blood samples with slight modification. In Drabkin's method, haemoglobin was estimated based on the principle that haemoglobin is oxidized to methemoglobin by potassium ferricyanide, that reacts with cyanide ions of potassium cyanide to form cyanmethemoglobin

1. 5ml of Drabkin's reagent was taken in a 15ml centrifuge tube
2. 20 µl of blood was added and mixed thoroughly
3. Kept at room temperature for 5 minutes and centrifuged at 5000rpm for 10 minutes
4. 2 ml of the resulting solution was placed into a cuvette
5. Optical density of the processed samples was read by using a spectrophotometer (Perkin Elmer Lambda 25 UV/Vis Spectrometer) at 540nm
6. Hb concentration of the samples was calculated using optical density of standard haemoglobin

Total Erythrocyte Count (TEC) and Total Leukocyte Count (TLC): Method described by Natt and Herrick (1952) was followed to estimate TEC and TLC.

TEC

1. Red blood cells were counted in a Neubaur's chamber (Haemocytometer) using Natt and Herrick's solution to obtain a 1:200 blood dilution (Varmaghany *et al* 2015)
2. Kept a coverslip on the ruled grid area with squares and placed a drop of diluted blood
3. The blood was allowed to spread evenly under the cover slip and kept for few minutes for settling of RBCs
4. Cells within 5 squares of the 25 squares in the middle of Neubaur's chamber (four squares at the four corners of the large square and square in the centre) were counted
5. Calculated with the equation mentioned below
   The number of red blood cells =N/5 x 25 x 200 x 10 (Al-Hassani 2011)

TLC

1. White blood cells were counted in a Neubaur's chamber (Haemocytometer) using Natt and Herrick's solution to obtain a 1:20 blood dilution (Varmaghany *et al* 2015)
2. Kept a coverslip on the ruled grid area with squares and placed a drop of diluted blood
3. The blood was allowed to spread evenly under the cover slip and kept for few minutes for settling of WBCs
4. Cells within 4 corner squares of Neubaur's chamber (four squares at the four corners of the large square and square in the centre) were counted
5. Calculated with the equation
   The number of white blood cells =N/4 x 20 x 200 x 10 (Al-Hassani 2011)

Differential Leukocyte Count (DLC):

1. Thin blood smears were prepared on a clean grease-free slides immediately after collection of blood
2. The smear was then air dried and fixed with methanol

3. After fixing, the smear stained with Leishman's stain
4. Hundred cells including heterophils, monocytes, lymphocytes, eosinophils and basophils were counted and recorded

3.1.1.3 Processing of blood samples for indirect ELISA

One ml of blood collected in blood collection vials (without anticoagulant) were used for serum collection. Vials containing blood were kept in a slanting position and blood was allowed to clot completely for 30-40 minutes. Then kept at 4°C overnight for complete oozing out of serum from the coagulated blood. Vials were centrifuged at 2500rpm for 5 minutes and serum collected were carefully transferred to a sterile 1.5ml microcentrifuge tube. Serum collected were stored at -80°C for further use.

3.1.1.4 Indirect ELISA for antibody detection of CIAV

The serum sample n=120 were tested for anti-chicken anemia virus antibody by using commercial ELISA kit (BioCheck CIAV antibody test kit, UK) by indirect method. Protocol followed asper the manufacturing company. In brief,

Reagents for Indirect ELISA

- *CAV Coated plates***:** Inactivated viral antigen on microtitre plates
- *Conjugate reagent*: Sheep anti-Chicken: Alkaline Phosphatase in Tris buffer with protein stabilisers, inert red dye and sodium azide preservative (0.1% w/v)
- *Substrate tablets*: PNPP (p-Nitrophenyl Phosphate) tablets dissolved in Substrate buffer.
- *Substrate buffer***:** Diethanolamine buffer with enzyme co-factors
- *Stop Solution*: Sodium Hydroxide in Diethanolamine buffer
- *Sample Diluent*: Phosphate buffer with protein stabilisers and sodium azide preservative (0.1% w/v)
- *Wash Buffer:* Powdered Phosphate Buffered Saline with Tween
- *Negative control:* Specific Pathogen Free serum in Phosphate Buffer with protein stabilisers and sodium azide preservative (0.1% w/v)
- *Positive Control*: Antibodies specific to CAV in Phosphate Buffer with protein stabilisers and sodium azide preservative (0.1% w/v)

Substrate Reagent Preparation

- one tablet of PNPP (p-Nitrophenyl phosphate) was added to 5.5 ml of substrate buffer and allowed to mix for 3 minutes or until fully dissolved. Used immediately after preparation

Sample Preparation and ELISA protocol

1. Each test sample was diluted to 1:500 ratio in sample diluent reagent
2. The precoated ELISA plates were loaded with prediluted samples (1:500), along with positive, negative and prediluted test serum in duplicates. (100 µl of negative and positive control were added to A1, B1 and C1, D1 respectively)
3. Incubated at room temperature and washed 4 times with wash buffer and dried by gentle tapping.
4. Added 100 µl of conjugate (Alkaline phosphatase) reagent to all the wells and covered the plates and Incubated at room temperature for 1hour.
5. Washed 4times and added 100 µl of substrate (p-Nitrophenyl phosphate) reagent to all the wells, covered the plates and Incubated at room temperature for 30minute
6. Added 100 µl of stop solution (Sodium hydroxide) to all the wells and recorded absorbance at 405nm within 30minutes
7. As per manufactures instruction, the ELISA test was valid when the mean negative control absorbance is <0.3 and difference between mean positive control and mean negative is control >0.15

Relative amounts of antibodies to CIAV in chicken serum samples were calculated as per manufactures instruction to get the endpoint results. Based on reference positive control, S/P ratio (sample to positive ratio) was calculated. The samples with S/P ratio of 0.35 or greater contains anti-CAV antibody and were considered positive

Calculation of S/P ratio

$$\frac{\text{Mean of test sample} - \text{Mean of negative control}}{\text{Mean of positive control} - \text{Mean of negative control}} = \mathbf{S/P}$$

Calculation of Antibody Titre

$\text{Log}_{10}\text{Titre} = 1.1 * \text{Log}_{10}(\text{SP}) + 3.361$

Antilog = Titre

3.1.1.5 DNA extraction from blood samples for molecular study

Total DNA was extracted from the seropositive whole blood samples using DNA extraction kit (HiPura™ Mammalian Genomic DNA Purification Kit, Himedia) as per manufactures protocol with slight modification. The protocol for DNA extraction is briefly described below.

1. Taken 100 µl of the fresh whole blood (at room temperature) in a 1.5 ml collection tube and added 100 µl of PBS to dilute the blood sample
2. Added 20 µl of the Proteinase K solution (20mg/ml) into the above collection tube containing blood. Vortexed (10-15 seconds) to ensure thorough mixing of the enzyme
3. Added 200 µl of the Lysis Solution to the sample, vortexed thoroughly for a few seconds to obtain a homogenous mixture. Incubated at 55°C in water bath for 30 minutes
4. Added 200 µl of C1 solution vortexed for 10-15 seconds Incubated at 70°C in water bath for 10 minutes
5. Added 200 µl of ethanol (chilled) to the lysate obtained from step 4 and mixed thoroughly by gentle pipetting for 5-10 seconds
6. Centrifuged at 3000xg for 5 minutes and carefully transferred the supernatant to spin column
7. Centrifuged the spin column at 10,000 rpm for 1 minute. Discarded the flow-through liquid and placed the column in a new 2.0 ml collection tube
8. Added 500 µl of wash solution to the spin column and centrifuged at 10,000 rpm for 1 minute. Discarded the flow-through liquid and re-used the same collection tube with the column.
9. Repeated the washing step and centrifuged at 16,000xg for 3minutes. Discarded the flow-through liquid and re-used the same collection tube with the column
10. Centrifuged for an additional one minute at 16,000xg to remove traces of Wash Solution.
11. Discarded the collection tube and placed the column in a new 2.0 ml collection tube
12. Added 30µl of the elution buffer directly into the column without spilling to the sides

13. Incubated for 5 minutes at room temperature and centrifuged at 10,000 rpm for 1 minute to elute the DNA
14. The DNA extracted (ng/µl) was measured and quantified (NanoDrop™, Thermo Fisher Scientific) spectrophotometrically and stored at -20°C for further use.

3.1.1.6Polymerase Chain Reaction (PCR) for Detection of CIAV

Optimization of PCR for amplification of VP3 gene

The total DNA extracted were amplified using published primers (Wani *et al* 2013) specific for VP3 gene region (364 base) of CIAV genome (Table 2).

Table 2: Detail of primers used for amplification of VP3 gene

S. No.	Primer ID	Sequence (5' to 3')	No. of base pair	Amplicon size	Reference
1	VP3 Forward	ATG AAC GCT CTC CAA GAA GAT A	22	364bp	Wani *et al* (2013)
2	VP3 Reverse	CAG TCT TAT ACA CCT TCT TGC G	22		

The following reaction components and condition used for PCR were optimized using PCR master mix (GoTaq green, Promega). A 25µl reaction volume was prepared in a 200 µl PCR tube (Table 3). A positive control with known template (DNA from CIAV vaccine strain VH/CAP/02), and a negative control with NFW were also kept along with sample during amplification to check the cross contamination in the PCR reactions. And incubation was carried as per Table 4.

Table 3: Detail of reaction components for PCR amplification

S. No.	Components	Amounts (µl)
1	PCR master mix (2x)	12.5
2	Forward primer (10pmol)	1
3	Reverse primer (10pmol)	1
4	Template DNA (.45µg)	3
5	Nuclease free water (NFW)	7.5
6	Total reaction volume	25

All the components were mixed properly (all work was done on cooler box) and briefly centrifuged to collect the total content at the bottom of the tube. Non-template control (NTC) and DNA from known positive were included in each PCR. The tubes were placed in the thermal cycler (BioRad, California) for amplification of the target genome.

Table 4: Thermal profile of PCR VP3

S. No.	Step	Temperature x Time	No. of cycles
1.	Initial denaturation	94°C-5min	1
2	Denaturation	94°C-1sec	35
3	Annealing	59°C-1sec	
4	Extension	72°C-1.5min	
5	Final Extension	72°C-10 min	1

3.1.1.7 Agarose gel electrophoresis for visualization of amplified PCR products

The PCR amplicons were visualized with agarose gel electrophoresis. 1.5 % agarose gel was prepared in 35ml of 1X TBE buffer.

1X Tris-borate-EDTA buffer (TBE)

Tris base	10.9 g
Boric acid	5.5 g
EDTA	0.93 g

Distilled water was added to make volume 1 litre (pH is 8.3)

The agarose in buffer was heated to dissolve and cooled to about 50°C. Ethidium bromide was added to a final concentration of 1µg / ml. Molten gel was poured into horizontal electrophoresis apparatus and allowed to solidify at room temperature. Combs were removed and electrophoresis tank was filled with 1X TBE to completely immerse the gel. Approximately 5µl of samples were loaded in to the wells along with the 100 bp marker (SMOBIO, Taiwan). The gel was electrophoresed at 90 V for 1 hr., visualized on a gel documentation system (Syngene, USA) and analyzed

3.1.1.8 Gel purification of VP3 gene specific band from PCR amplified product

Approximately 20µl PCR amplicon were loaded to 1.5% gel and electrophoresed as per method previously described. The VP3 gene specific band was

cut carefully with a sterile sharp scalpel blade by exposing the gel on a transilluminator (minimum exposure of gel to UV to avoid nicking of the DNA). Cut gel were collected in a sterile 1.5ml microcentrifuge tube. PCR product were purified using a gel extraction kit (Wizard® SV Gel and PCR Clean-Up System, Promega) as per the manufacture's protocol.

1. The excised gel was placed in to a pre-weighed sterile 1.5ml microcentrifuge tube and weighed
2. Equal volume of binding buffer was added to microcentrifuge tube and incubated at 55-60°C for 10-15minutes with intermittent mixing (or until the gel slice has completely dissolved). Centrifuged the tube briefly.
3. Transferred the dissolved gel mixture the SV Mini column assembly and incubated for 1 minute at room temperature.
4. Centrifuged the SV Mini column assembly in a microcentrifuge at 16,000 × g (14,000rpm) for 1 minute at room temperature and discarded the flow through
5. 700µl of Membrane Wash Solution was added to the Mini column and centrifuged for 1 minute at 16,000 × g (14,000rpm) at room temperature and discarded the flow through
6. Repeated the washing step with 500µl of Membrane Wash Solution, and centrifuged for 5 minutes at 16,000 × g at room temperature.
7. An empty spin was done for 1minute at 16,000xg at room temperature to remove any residual ethanol from the membrane
8. Transferred the SV Mini column to a clean 1.5ml microcentrifuge tube and applied 30µl of Nuclease-Free Water directly to the centre of the column without touching the membrane with the pipette tip.
9. Incubated at room temperature for 5 minute and centrifuged for 2 minutes at 16,000 × g (14,000rpm)
10. The eluted DNA was checked for quantity and quality spectrophotometrically (NanoDrop™, Thermo Fisher Scientific) and by agar gel electrophoresis
11. stored at –20°C till further use.

3.1.1.9 Sequencing and Phylogenetic analysis of VP3 gene

Purified DNA was sent for commercial sequencing by Eurofins Genomics India Private Ltd., Bangalore. The data obtained were analyzed by Lysergene DNASTAR and MEGA7.0 software. The VP3 DNA sequences of CIAV were aligned

with other published sequences (retrieved from GenBank, NCBI) using ClustalV method of MegaAlign programme (DNASTAR, US) and Phylogenetic analysis was done using Maximum likelihood method (MEGA7, USA.)

3.1.1.10 Statistical analysis

The haematological, serological and molecular data obtained were statistically compared using Tukey's one-way ANOVA and the results were documented.

3.1.2 Tissue samples collected for detection and isolation of CIAV

The tissue samples *viz.* Thymus, Liver, Bone marrow, Spleen, Bursa was collected from dead birds submitted for post-mortem examination in the Department of Veterinary Pathology, COVS, GADVASU, Ludhiana as well as collected from the private clinics/farms located in the various regions of Punjab state (Table 5).

Table 5: Detail of tissue sample collection from chicken

S. No.	Place	Number of Organized Farms	Number of birds
1.	Fazilka	2	2
2.	Ludhiana	3	27
3.	Moga	3	9
4.	Ferozepur	1	2
5.	Hoshiarpur	1	8
6.	Mansa	1	3
7.	Barnala	1	2
8.	Jalandhar	1	2
9.	Mohali	2	6
10.	Sangrur	1	4
	Total	**16**	**65**

Tissue samples collected in sterile Phosphate Buffered saline (PBS-pH 7.2 to 7.4) were stored at -80°C for further molecular detection and isolation of CIAV.

Phosphate Buffer Saline (PBS, pH=7.2, 1X)

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	200 mg
Potassium dihydrogen phosphate (KH_2PO_4)	240 mg

Disodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$)	1.150 g
Distilled water	1 litre

Autoclaved at 15 lbs. pressure for 15 minutes and kept at 4^oC till use.

3.1.2.1 Processing tissue samples for molecular detection and isolation of CIAV

1. Tissue samples i.e. Thymus, Liver, Bone marrow, Spleen, Bursa were collected in sterile sample collection vials with PBS (pH 7.2)
2. Each tissue was washed twice with sterile PBS
3. Triturated and homogenized with sterile sand, mortar and pestle
4. PBS was added to prepare 10% (w/v) suspension and transferred to sterile 15ml centrifuge tube
5. Centrifugation was carried out at 2500xg at 4°C for 20minutes (REMI, India) and supernatant was transferred to another sterile15ml tubes.
6. Repeated the step 5 twice and supernatant was aliquoted into 1.5ml sterile microcentrifuge tube after filtering through 0.22µm membrane filter (Corning, Germany)
7. Added antimicrobial solution (Streptopencillin, 1X, Himedia) at 1% level to each inoculum prepared, sealed and incubated at 37°C for 45 minutes
8. The inoculums were stored at -80°C until further use.

3.1.2.2 DNA extraction and PCR

DNA was isolated from each tissue inoculums (Thymus Liver, Bone Marrow, Spleen, and Bursa) using DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN, Germany) as per the manufactures protocol with slight modification. In brief,

1. Taken 200µl of the inoculum (at room temperature) in a 1.5 ml collection tube
2. 20µl Proteinase K solution (20mg/ml) was added into the above collection tube containing inoculum. Mixed by Vortexing (10-15 seconds)
3. Added 200µl of AL buffer and vortexed for 10-15 seconds. Briefly centrifuged to bring the content towards bottom.
4. Incubated at 56°C in water bath for 10 minutes and after incubation, samples were vortexed for 15 seconds and briefly centrifuged
5. Then added 200µl of ethanol (96-100%) and the contents were again mixed by vortexing.

6. The mixture was transferred into DNeasy mini spin columns placed in 1.5ml collection tubes and centrifuged at 6000 x g for 1 min.
7. Discarded the flow through along with collection tube and then DNeasy mini spin columns were placed into new 1.5 ml collection tubes.
8. Washing was done by adding 500µl of AW1(wash buffer 1) to mini spin column and centrifuged at 6,000 x g for 1 min.
9. Discarded the flow through along with collection tube and then DNeasy mini spin columns were placed into new 1.5 ml collection tubes.
10. Repeated step 8 with 500µl of AW2 (wash buffer 2) and centrifuged at 14,000 x g for 3minutes and flow through along with collection tube was discarded.
11. Columns were placed into new 1.5ml collection tube and centrifuged at 14,000 x g for 1minutes to remove any remaining wash buffer in the membrane.
12. DNeasy mini spin column were placed into a new sterile 1.5ml microcentrifuge tube and added 30µl of AE buffer (Elution buffer) to each sample directly onto the DNeasy membrane.
13. The samples were incubated at room temperature for 5 minute and centrifuged at 6,000 x g for 1 min (optional: repeated step 13 once more to elute any remaining DNA on the membrane).
14. The DNA eluted (ng/µl) were measured spectrophotometrically (NanoDrop™, Thermo Fisher Scientific) and stored at -20°C for further use.

Optimization of Polymerase chain reaction (PCR) for amplification of VP1, VP2 and VP3 gene

The total DNA extracted were amplified using published primers (Wani *et al* 2013) specific for VP1, VP2 and VP gene of CIAV genome (Table 6). The reaction components and condition used for PCR amplification were optimized as per the procedure described earlier in this chapter Table 3 and Table 4.

Table 6: Detail of primers used for amplification of VP1, VP2 and VP3 genes

S. No.	Primer ID	Sequence (5' to 3')	No. of base pair	Amplicon size	Reference
1	VP1 Forward	ATG GCA AGA CGA GCT CGC	18	1.35kp	(Wani *et al* 2013)
2	VP1 Reverse	TCA GGG CTG CGT CCC CCA	18		
3	VP2 Forward	ATG CAC GGG AAC GGC GGA C	19	652	
4	VP2 Reverse	TCA CAC TAT ACG ACC GGG G	19		
5	VP3 Forward	ATG AAC GCT CTC CAA GAA GAT A	22	362	
6	VP3 Reverse	CAG TCT TAT ACA CCT TCT TGC G	22		

3.1.2.3 Visualization of amplified PCR products by agarose gel electrophoresis

The PCR amplicons were visualized with agarose gel electrophoresis. 1% agarose gel was prepared in 35ml of 1X TBE buffer. The agarose in buffer was heated to dissolve and cooled to about 50°C. Ethidium bromide was added to a final concentration of 1µg / ml. Molten gel was poured into horizontal electrophoresis apparatus and allowed to solidify at room temperature. Combs were removed and electrophoresis tank was filled with 1X TBE to completely immerse the gel. Approximately 5µl of samples were loaded in to the wells along with the 100 bp marker (SMOBIO,Taiwan). The gel was electrophoresed at 90V for 60minutes, visualized on a gel documentation system and analyzed.

3.1.2.4 Sequencing of VP1, VP2 and VP3 gene of CIAV

Specific DNA band of each gene were gel purified as per the protocol described earlier in this chapter. Forward and reverse sequencing of DNA was done by commercial out sourcing from (AgriGenome, Kerala). The data obtained were analyzed using Lysergene DNASTAR and MEGA7.0 software (Kumar *et al* 2016). The deduced sequences were submitted to National centre for Biotechnology Information (NCBI) and accession numbers were obtained.

The VP1 VP2 and VP3 DNA sequences of CIAV were aligned with other published sequences available in Databank of NCBI using ClustalW method of MegaAlign programme and Phylogenetic analysis was done using Maximum Likelihood method (MEGA7, USA.)

3.1.3 Isolation of CIAV in Cell culture

3.1.3.1 Materials required for virus isolation in cell culture

MDCC MSB1 Cell line

The MDCC MSB1 cell line maintained in the Venkateshwara hatchery, Pune, Maharashtra was procured for isolation of CIAV from field samples. Cells were propagated in RPMI-1640 medium (Himedia, Mumbai, India) supplemented with foetal bovine serum (Gibco, USA). Embryonated Chicken Egg (ECE) were procured from Venkateshwara hatchery, Ludhiana, Punjab.

3.1.3.2 Plasticwares, Media, Buffers and Reagents used for cell culture

Cell culture flask, cell culture plates Nunc (Denmark), filter tips of various volume (Corning, Germany), pipettes of variable volume. RPMI-1640 medium,

sodium bicarb, L-glutamine, sodium pyruvate, foetal bovine serum (Himedia, Mumbai, India) Antimicrobial solution (Himedia, Mumbai, India) cell freezing medium, Phosphate buffered solution, triple distilled water, CO_2 incubator, inverted microscope. Wherever necessary, molecular biology grade chemicals/biochemicals were used.

3.1.3.3 Preparation of RPMI-1640 medium

Prepared 1X RPMI-1640 medium (Sigma, USA) as per manufactures recommendation. Added one vial of powdered medium into one litre of triple distilled autoclaved water. Added 292mg of L-glutamine (2mM final concentration) and HEPES 0.1ml (10mM final concentration) to 1L of RPMI-1640 medium. Sterilization has been done using 0.22micron filter and sterility checked by keeping at 37°C for 24 hours. Then kept at 4°C till further use. Working solution of RPMI-1640 medium was prepared by adding 10% FBS and 1% antimicrobial solution and maintenance medium by adding 2-3% FBS.

3.1.3.4 Sub-culturing MDCC MSB1 cell line

MDCC MSB1 cells were sub cultured in a tissue culture flask($25cm^2$) (corning, Germany) Cells obtained from Venky's laboratory were split in to two and allowed to grow in growth medium for 24hours at 37°C and 5% CO_2. At 60-80% confluency cells were harvested and number of viable cells were calculated by using Trypan blue dye exclusion method. Number of viable cells/ml were determined before freezing of cells for long term storage.

3.1.3.5 Determination of average cells /ml

1. Trypan blue dye exclusion method used to calculate number of viable cells using haemocytometer
2. 50µl of cell suspension was mixed with 50µl of trypan blue dye (Thermoscientific, USA).
3. Loaded approximately one drop to the Neubauer's chamber (Marienfeld) and kept for 1minute for settling of the cells
4. Total number of viable cells in the 4 large square were counted and calculated average number of cells per square
5. Concentration of viable cells/ml were calculated by following equation
   Average number of cells/ square X Dilution factor X 10^4

3.1.3.6 Cryopreservation Media

CryoXL Cell freezing medium with added DMSO (1X) and foetal bovine serum (FBS) procured from Himedia was used in the study.

3.1.3.7 Freezing of cell line for Cryopreservation

Taken cell suspension in to 15ml sterile tube (corning, Germany) and centrifuged at 600xg for 5-10 minutes. Supernatant were discarded and the cell pellet were gently dissolved in 50-100µl RPMI-1640 medium. Slowly added freezing medium and mixed the cells gently. Aliquoted 1ml of the cells in to sterile marked cryovials and kept for gradual cooling (30minutes at 4 °C then 2-3hours in -20°C) and then kept overnight at -80°C. Next day transferred the vials into gas phase of liquid nitrogen (LN_2) container and kept for overnight. Finally, on the very next day, the cryovial were kept in the liquid phase of LN_2 container.

3.1.3.8 Virus isolation

PCR positive samples were used to isolate the CIAV by co-cultivation method in MDCC MSB1 cell line described by (Noteborn *et al* 1994a) with slight modifications. Virus inoculum kept at -80°C were processed by freeze-thaw method. The refreshed inoculum was filtered through .22-micron syringe filter before infecting the cells.

1. From a $25cm^2$ flask, MDCC- MSB1 cells with about 75-80% confluency was transferred to 15ml sterile tube and centrifuged at 600xg for 5 -10 minutes.
2. Supernatant were removed and resuspended in RPMI-1640 without antibiotic solution and serum to make a cell suspension of 1.5 to 2 x 10^5 cells/ ml (calculated by dye exclusion method).
3. 0.5ml cell suspensions were Centrifuged at 600xg for 5-10 minutes and discarded the supernatant and Pellets were resuspended in 0.5ml of filtered inoculums. Transferred to 12 well plate (Nunc, Denmark) and gently taped for uniform distribution of cells.
4. The plate was incubated in a CO_2 incubator (37ºC, 5% CO_2 and humidity) for 1 hours with intermittent shaking for the adsorption of virus.
5. After adsorption, the volume was adjusted to 2ml/ well with maintenance medium (RPMI-1640 with 2% FBS) and kept at 37 ºC and 5% CO_2. Negative control cell also maintained during entire process of isolation

6. Blind passages were carried out at 2-3days interval.
7. Daily observed for characteristic cytopathic effects (CPE) after each blind passage (Ballooning, enlargement, granulation and breakage of the affected cells).
8. The culture showing Characteristic CPE were harvested and preserved at -80 ºC for further use.

3.1.3.9 Confirmation of cell culture isolates of CIAV

Confirmation by VP1 and VP3 gene specific PCR

Confirmation of isolated virus were done by diagnostic PCR. DNA were extracted from the cell culture isolates using commercial DNA extraction Kit (Qiagen, Germany) method already explained earlier in this chapter. Extracted DNA were amplified by PCR method using VP1 and VP3 gene specific primers and visualized by agar gel electrophoresis (described the methodology earlier in this chapter). DNA from uninfected MDCC MSB1 cells were kept as negative control.

3.1.3.10 Confirmation by immunofluorescent assay

1. MDCC- MSBI cells in log phase (24-36 hours culture) in 12 well plates were infected with CIAV isolate.
2. After the observation of characteristic CPE, infected cells were harvested and prepared thin smear on positively charged microscopic slides and dried at room temperature. Maintained uninfected MDCC MSB1 as negative control throughout the test
3. Permeabilized and fixed with chilled methanol and acetone (i.e. 1 parts methanol and 1-part acetone) and kept at -20 ºC for 20 minutes.
4. Washing done 4times with PBS-tween 20
5. The fixed cells were incubated with 100µl of anti- CIAV antibody (diluted 1:100 in PBS) at 37 ºC for 1 hour.
6. After washing three times with washing buffer,
7. antibody bindings were detected by adding 100 µl of goat anti-chicken IgG antibody conjugated with FITC **(**Invitrogen,) (diluted 1part in 10,000 parts of 1X PBS) followed by 45 minutes incubation at 37 ºC.
8. washed with distilled water for 10-15 minutes
9. Then 50µl of mounting media (50% glycerol in PBS) were added after washing the conjugate with PBS.

10. Finally, they were visualized under fluorescence microscope (Nikon Eclipse Ts 100) at 100 X magnification.
11. The results were recorded by taking the image.

3.1.3.11 Demonstration of CIAV particle by electron microscopy

CIAV isolated in MDCC MSB1 were send for electron microscopic imaging from Electron Microscopy & Nanoscience Laboratory, Department of Soil Science, Punjab Agricultural University, Ludhiana, Punjab.

To demonstrate cell culture isolates of CIAV, the cell culture supernatant was negatively stained with phosphotungstic acid. 2% phosphotoungstic acid was prepared and filtered through 0.2micron filter 4-5times. One drop of virus suspension was loaded on copper grid and kept for 15 minutes. Excess fluids were removed by filter paper and grid was loaded with 2% phosphotoungstic acid for few seconds. The grid was dried and were examined in electron microscope (Hiachi H-7650)

3.1.3.12 Optimization of PCR for partial/whole genome of CIAV and sequencing

A PCR was optimized for amplifying whole genome of CIAV. The total DNA was isolated from each cell culture isolates (n=10) CIAV. Detail of the primer used in this study are given in the Table 7. A total volume of 50µl reaction mixture was prepared by using Q5 High-fidelity PCR mix (New England Biolabs, US). Time, Temperature, concentration of various in gradients were optimized and used in the present study were given in the Table 8.

Table7: Detail of primers used for amplification of whole genome of CIAV genome

S. No.	Primer ID	Sequence (5' to 3')	No. of base pair	Amplicon size	Reference
1	WG Forward	GAA TTC CGA GTG GTT ACT ATT CCA TCA	27	2.3kb	(Krishan *et al* 2015)
2	WG Reverse	GAT AGT GCG ATA AAT CTA TTT TCT GCG T	28		

Table 8: PCR components used for amplification of whole genome of CIAV

S. No.	Components	Amounts (µl)
1.	Q5 HiFi reaction buffer (5x)	10.0
2.	dNTPs mix (10 mM each)	1.0
3.	Forward primer (10pmol)	2.0
4.	Reverse primer (10pmol)	2.0
5.	Q5 high fidelity DNA Polymerase (2U/ µl)	0.5
6.	Template DNA (.5µg)	3.0
7.	Nuclease free water (NFW)	31.5
8.	Total reaction volume	50.0

All the components were mixed properly (all work was done on cooler box) and briefly centrifuged to collect the total content at the bottom of the tube. Non-template control (NTC) and DNA from known positive cell culture isolate were included in each PCR to check the cross contamination in the PCR reactions. The tubes were placed in the thermal cycler (BIO RAD, thermal cycler gradient,) for amplification of the target genome (Table 9).

Table 9: Thermal cycling profile of PCR

S. No.	Step	Temperature x Time	No. of cycles
1.	Initial denaturation	94°C-4min	1
2	Denaturation	94°C-1 min	35
3	Annealing	54°C-1 min	
4	Extension	72°C-2min	
5	Final Extension	72°C-10 min	1

3.1.3.13 Confirmation of PCR amplified whole genome of CIAV

The whole genome of CIAV amplified by PCR were confirmed by gel electrophoresis (0.75% gel) and specific DNA band representing genome of CIAV were gel purified by Promega gel kit as per the manufactures protocol described earlier in this chapter. Purified products (ng/µl) were measured and quantified by Nanodrop spectrophotometer and recorded for further use.

3.1.4 Cloning and sequencing of whole genome of CIAV

3.1.4. 1 Media and buffers used for cloning

Lysogenic Broth (LB medium

LB broth (Himedia) dehydrated	12.500 gm
Distilled water up to	500 ml

Sterilized by autoclaving and Stored at 4ºC.

LB Agar

L B agar (Himedia) dehydrated	40 gm
Distilled water up to	1000 ml

Sterilized by autoclaving. Cooled down at 45ºC and supplemented with filtered ampicillin

(100µg/µl) before platting. Plates were stored at 4ºC.

SOC medium

Sterile SOB	99.00 ml
Sterile D-Glucose (2M)	1.00 ml

Stored at 4ºC.

0.1M IPTG solution

IPTG	1.2
Autoclaved distilled water	50ml

Filter sterilized and stored at 4ºC

X gal

X gal	100mg
Dimethyl Formamide	2ml

Covered with aluminium foil and stored at 4ºC.

3.1.4.2 Cloning of Purified whole genome specific DNA in pGEM-T Easy Vector

The gel purified DNA were A-tailed (Table 10) and cloned into pGEM®-T Easy vector (Promega, Madison, USA) with 1:3 vector to insert ratio was followed by T-A cloning strategy (Lambden *et al* 1992) according to the Manufacturer's protocol as described below

Table 10: A-tailing of purified PCR products

S. No.	Component	Amount (µl)
1	10X buffer	2.5
2	100mM dATP	0.5
3	50mM $MgCl_2$	0.5
4	Taq polymerase	0.25
5	PCR products (Purified)	20
6	NFW	1.25
7	Total	25

Vector: Insert ratio calculation

$$\frac{\text{Vector concentration (ng)} \times \text{insert size (Kb)}}{\text{Vector size (Kb)}} \times \text{(Insert: Vector ratio)} = \text{Insert quantity (ng)}$$

The kit components (pGEM®-T easy linear vector, 2X rapid ligation buffer and T4 DNA ligase) were thawed on ice and mixed properly by flicking. The tubes were centrifuged briefly to collect the contents at the bottom of the tube. Ligation reactions were carried out at final volume of 10µl in a 200µl sterile PCR tubes. The reaction mixture was prepared by using following component described in the (Table 11).

Table 11: Ligation reaction components for cloning

S. No.	Reaction Components	Amount (µl)
1.	2X Rapid ligation buffer	5.0
2.	pGEM-T Easy Vector (50 ng/µl)	1.0
3.	Purified PCR product (45-50 ng/ µl)	2.5
4.	T4 DNA ligase (3weiss U/µl)	1.0
5.	Nuclease Free Water	0.5
6.	Total	10.0

The reaction components were mixed gently by flicking and then centrifuged briefly to collect the tube content at the bottom. The tubes were incubated at 4°C overnight for better ligation. The ligated product (2µl) was transformed into chemically competent *E. coli* (JM109) cells for selection of recombinant bacterial colony and the remaining was stored at -20°C for further use.

3.1.4. 2 Transformation of ligated plasmid vector into competent *E. coli* cells

Chemically competent *E. coli* cells were transformed with ligated plasmid vector by heat shock method

1. The ligated plasmid vector was mixed by gentle flicking and briefly centrifuged to collect the content at the bottom of the tube.
2. Competent cells were briefly thawed on ice and 2 µl of individual ligated product was added in to the respective tubes containing 50µl of competent cell suspension. Then mixed properly by gentle flicking and incubated on ice for about 20 minutes.
3. The cells were then subjected to heat shock at 42°C for 90 seconds and immediately snap cooled on ice. Cells at this stage were handled very carefully without any shaking
4. Added 900µl of pre-warmed SOC medium slowly to the tube containing the transformed bacterial cell suspension and mixed properly
5. The bacterial cells were allowed to grow at 37°C in an orbital shaker incubator at 170 rpm for 1 and 1/2 hour
6. LB agar plate containing ampicillin were supplemented with 20 µl of X-gal and 100 µl of IPTG and allowed to absorb at 37°C for 30 minutes.
7. 200µl of bacterial culture was spread uniformly on the LB agar plate with a smooth and sterile L-loop spreader
8. The plates were kept for 30 minutes at up-side-up position for absorption of excess culture fluid and finally incubated at 37°C for overnight at up-side-down position for growth of bacterial colonies
9. Next day, the plates were checked for blue-white colonies (Blue-nonrecombinant and white-recombinant)

   Suitable white colonies of recombinant bacteria were marked and further screened for presence of specific gene insert and the culture plates were stored at 4°C after sealing and wrapping for protection from light and other contamination.

3.1.4. 3 Screening the Recombinant Colonies for Insert

Recombinant colonies were initially screened by blue white screening and followed by colony-PCR using gene specific primer

3.1.4. 4 Colony-Polymerase chain reaction (colony-PCR)

Colony-PCR was performed using the same set of primers used to amplify the whole genome of CIAV. Initially white colonies were marked and selected for colony PCR. The PCR reaction components (replacing DNA with colonies as template) and PCR profile were described earlier in this chapter. The PCR reactions were carried out in a final volume of 25µl in following steps.

3.1.4. 5 *Processing of colonies for PCR*

The PCR positive colonies (3-4) were randomly selected and picked with a sterile tooth pick. Then inoculated into 5 ml of LB broth (with ampicillin @ 100mg/ml) and allowed to grow overnight at 37ºC in an orbital shaker incubator at 170rpm.

1. Approximately 200µl overnight grown bacterial cells were taken in 1.5ml microcentrifuge tubes.
2. Centrifuged at 11000xg for 5 minutes to remove supernatant and the pellet was resuspended in 20µl Nuclease free water.
3. Incubated at 95 ºC for 10 minutes and immediately transferred to -20 ºC kept for freezing the contents
4. Once freeze, tubes were removed from the -20 ºC and thawed at room temperature.
5. The tubes were then centrifuged at 2500xg for 5 minutes and supernatant was collected into a sterile 200µl tubes.
6. From the above collected supernatant, 3µl was added to the PCR master mix (Table.12).

Table 12: PCR master mix for colony PCR

S. No.	Components	Amounts (µl)
1	PCR master mix (2x)	12.5
2	Forward primer (10pmol)	1
3	Reverse primer (10pmol)	1
4	Colony supernatant	3
5	Nuclease free water (NFW)	10.5
6	Total reaction volume	25

7. The tubes were briefly centrifuged to collect the content at the bottom of the tubes. Suitable negative control tube containing fresh LB broth with ampicillin was also kept
8. PCR reaction was carried out with the following cyclic condition (Table.13)

Table 13: Thermal profile for colony PCR

Sl. No.	Step	Temperature x Time	No. of cycles
1	Initial denaturation	95°C-4min	1
2	Denaturation	95°C-30sec	35
3	Annealing	59°C-30 sec	
4	Extension	72°C-1min	
5	Final Extension	72°C-10 min	1

The PCR amplicon were resolved in 0.75% agarose gel and electrophoresis was carried as described earlier in this chapter.

3.1.4. 6 Plasmid DNA extraction

The PCR positive colonies (3-4) were randomly selected and picked with a sterile tooth pick. Then inoculated into 5 ml of LB broth (with ampicillin @ 100mg/ml) and allowed to grow overnight at 37ºC in an orbital shaker incubator at 170rpm. Plasmid DNA was extracted by using NucleoSpin Plasmid DNA purification kit (Macherey-Nagel, USA) following manufacturer's protocol as mentioned below.

1. Approximately 5 ml of overnight grown bacterial culture was taken into a sterile micro centrifuge tube and centrifuged at 11,000xg for 30s at room temperature. Bacterial cell pellet was harvested by discarding the supernatant
2. Added 250µL of Buffer A1 and resuspend the cell pellet completely by vortexing
3. Added 250µL of Buffer A2 and mixed gently by inverting the tube 6–8 times. Do not vortex to avoid shearing of genomic DNA
4. Added 300µL Buffer A3 and mixed thoroughly by inverting the tube 6–8 times until blue samples turn colourless completely
5. Centrifuged for 5 min at 11,000 x g at room temperature
6. Placed a NucleoSpin® Plasmid Column in a collection Tube (2 mL) and pipetted from step 3, a maximum of 750µL of the supernatant onto the column

7. Centrifuged for 1 min at 11,000 x g and discard flow through and placed the Column back into the collection tube.
8. Added 500µl Buffer AW, optionally preheated to 50 °C, and centrifuged for 1 min at 11,000 x g and discard flow through and placed the Column back into the collection tube
9. Added 600µl Buffer A4 and centrifuged for 1 min at 11,000 x g. Discarded flow the through and placed the NucleoSpin® Plasmid column back into the empty collection tube.
10. For drying the column, centrifuged for 2 min at 11,000 x g and discard the collection tube.
11. Placed the NucleoSpin® Plasmid column in a 1.5 ml microcentrifuge and added 50µl Buffer AE. Incubated for 5 min at room temperature. Centrifuged for 1 min at 11,000xg.
12. These plasmid preparations were used for PCR analysis and stored at -20ºC for further use and sequencing of insert

3.1.4.7 Sequencing of partial/whole gnome of CIAV

Forward and reverse sequencing of the cloned genome of CIAV in plasmid vector was carried out by commercial out sourcing (AgriGenome, Kerala)

3.1.4.8 Molecular characterization of partial/ whole genome of CIAV

The sequence data generated from DNA of CIAV were analyzed by using DNASTAR (Lasergene®) software to obtain the full-length nucleotide sequence of the CIAV genome. Basic Local Alignment Search Tool (BLAST, NCBI) used to check the initial identification and further analyses were carried out by using different software packages/programs.

3.1.4.8 Phylogenetic Analysis

The nucleotide sequences of CIAV isolates generated in the current study and the nucleotide sequences of complete genomes available in the public database (NCBI/GenBank) was analyzed phylogenetically to find out the identity/divergence of each isolates. Multiple sequence alignments were performed using Clustal V/ClustalW algorithm and phylogenetic tree was generated by using MEGA5.10 software (Tamura *et al* 2011). The evolutionary history was inferred using the Maximum Likelihood (ML) method and the bootstrap consensus tree was inferred from 1,000 replicates to represent the evolutionary history of the taxa analyzed.

CHAPTER – IV

RESULTS AND DISCUSSION

Chicken Infectious anemia is one of the important emerging viral disease of chicken which pose severe threat to the poultry sector in India. Because of the important role in immunosuppression and suppression of vaccinal immunity, CIAV (along with various other avian viruses like MDV, ALV, NDV, IBDV, IBV etc.) became the serious burden for the poultry farmers. Keeping in view these factors in mind, the present study was planned for a thorough systematic investigation of CIAV among poultry dense population of Punjab state in India. A serological and molecular study was conducted on samples collected from various regions of Punjab and phylogenetic relation with various other Indian and International isolates were analyzed.

4.1 The seroprevalence of CIAV

4.1 .1 Health status of the birds included in the serological study

The study was carried out on 120 blood samples collected from 27 different chicken flocks covering 12 different geographical area of Punjab. All samples were from either apparently healthy or unhealthy/ill birds. Neither of them was vaccinated against CIAV. All of them were raised in conditions of intensive production with food and water provided *ad libitum* (Figure 4). Production loss, retarded growth and history of previous mortality were observed in farms of apparently healthy birds. Clinical signs noticed in unhealthy/ill bird were dullness, droopiness, respiratory distress, birds under treatment of other microbial infection and sudden death /mortality of birds in the flock. Broilers and layers both were included in the present study. Acute mortality was observed in some layer farms during the peak summer season. The blood sample was collected from different age group ranging from one week to greater than 8 weeks. During the time of collection few samples showed change in consistency of blood (very thin blood with reduced viscosity, increased clotting time etc.). Whole blood and serum were collected and a comparative study was done on haematology and serology for screening of antibody to CIAV expected to be from either vertical transmission or from natural infection.

Previous serological studies carried out in the different part of the world to screen the birds for the presence of antibody to CIAV from natural infection. The birds with history of poor performance, production loss, poor immune response to other avian vaccine are suggestive of CIAV presence in the flock (Ledesma *et al* 2001, Bidin *et al* 2010, Bhatt *et al* 2011). As the virus is having the feature of immunosuppression and subclinical nature, co-infection with other viruses may aggravate the outcome of CIAV infection. Several studies have confirmed the virus persistence and subclinical nature of CIAV.

Farhoodi *et al* (2007) reported the subclinical nature of the virus among apparently healthy chicken flock in Iran. They observed hemorrhages in apparently healthy bird which slaughtered for meat purpose. Hadimli *et al* (2008) showed high prevalence of subclinical CIAV infection in the unvaccinated chicken flocks in Turkey. McIlroy *et al* (1992) reported the outbreak of CIA in broiler flock was due the virus circulating sub clinically in breeder flocks which caused significant economic loss due to lower weight gain and high mortality in broiler chicken. De Herdt *et al* (2001) conducted serologic survey in unvaccinated broiler flocks and demonstrated seroconversion against chicken infectious anemia virus (CIAV) in all parent flocks examined at slaughter age. Results from their study indicated the high prevalence of CIAV in both broiler parent and broiler flocks which increased slaughterhouse condemnation. Results of the present study corroborate with earlier findings as none of the flock was vaccinated against CIAV.

4.1.2 Analysis of serological and Haematological changes

Detection of anti-CIAV antibody was done by indirect ELISA protocol (Figure 5). Haematological parameters like packed cell volume (PCV), haemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC) *etc.* were analyzed. Out of 120 serum sample analyzed 66 (55%) were found positive for anti-CIAV antibody (Figure 6) and others were negative (45%). Out of 91 apparently healthy birds, 51 (56.04%) were found positive for anti-CIAV antibody. A total of 29 clinically ill birds screened, 15 (51.7%) were found positive for CIAV antibody. Among the total broiler (87) and layer birds (33) screened 36 (41.37%) and 30 (90%) were found positive for chicken anemia virus

Fig. 4: Blood collection from apparently healthy birds. A) Intensive/cage raring system followed in organized farm. B) sample collection from the wing vein

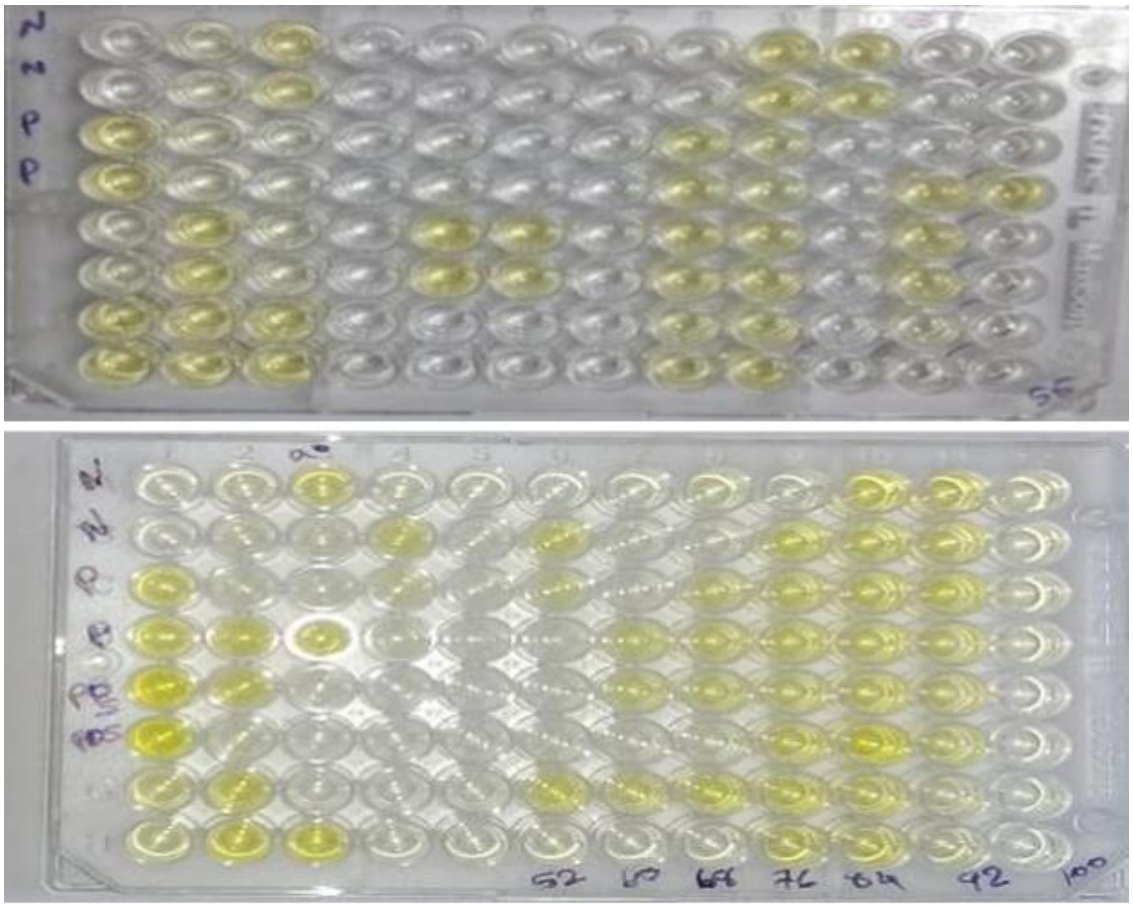
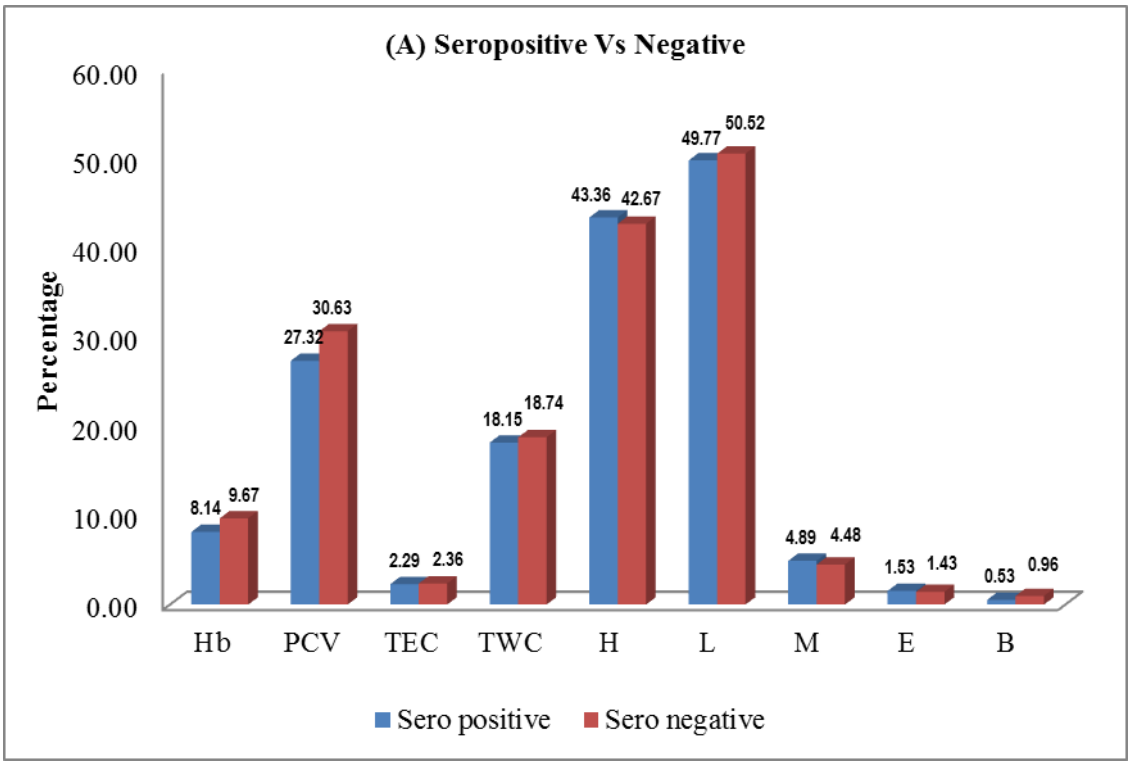


Fig. 5: Indirect ELISA for detection of anti-CIAV antibody in serum samples. An yellow colour development indicates positive test and no colour development at the end of test indicate negative



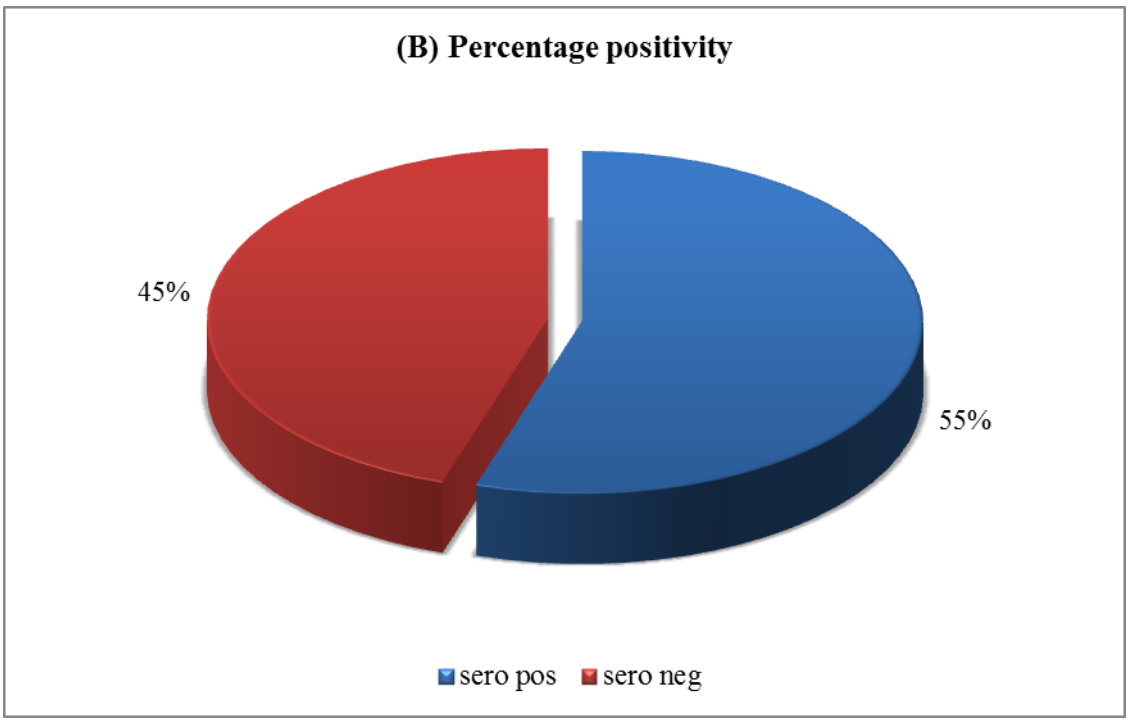


Fig. 6: Comparative blood profile of seropositive versus seronegative birds for CIA virus. A) The graph shows a change in Haemoglobin, Packed Cell Volume and Lymphocytes. B) Percentage positivity of anti-CIAV antibody in samples screened for CIAV presence

respectively (Table 14). Also, there was a significant change (P<0.01) in PCV, TEC, Hb were observed among flocks tested for anti-CIAV antibody (Table 15 to Table 20).

In the previous studies, similar findings were reported from the various part of the world including India (Owoade *et al* 2004, Bidin *et al* 2010, Bhatt *et al* 2011, Smock *et al* 2012). The results from their study showed a high prevalence of CIAV in unvaccinated bird. Which clearly indicate that virus is circulating among the chicken flocks either clinically or sub-clinically. Ledesma *et al* (2001) suggested that lower productivity and poor response to vaccination may be related to CIAV. Maternal antibody may provide protection at younger age but as it diminishes, the bird may become susceptible to infection but not disease. Bhatt *et al* (2011) reports a high seroprevalence of CIA (79.57% in Punjab) in northern part of India.

Toro *et al* (2006) conducted a retrospective serological study on serum sample which were maintained in an experimental chicken farm at Auburn University's Department of Poultry Science from 1959 to 2005. Their study demonstrated that CIAV antibodies were detected in sera from all years tested and provide serologic evidence that CAV must have been in the field even before its first isolation in 1979. Also, a seroprevalence of 54.5% were observed in fresh sample collected during 2005. Sharma *et al* (2014) showed an overall seroprevalence of 92.9% in layers and 58.3% in broilers in samples collected from Grenada, West Indies.

Baksi *et al* (2016) conducted seroprevalence study in six different states of India and found out an overall prevalence of 39.3% in apparently healthy birds with high prevalence was observed in age group between 30 to 50 weeks.

Shettima *et al* (2017) report that antibody to CIAV was observed not only in chicken but also in other poultry species like turkeys (23.6%), ducks (13.7%) and geese (22.7%) which were raised together alongside the chickens.

Previous reports of high seroprevalence throughout the world clearly indicate the emerging status of CIA. Therefore, the results from the present serological study confirm the presence of CIAV circulating among various flocks in Punjab state. All farms screened were found positive for CIAV antibody. In India regular vaccination of birds with CIAV is not practiced. Since all the samples were from unvaccinated

birds, they might have got infection either from natural exposure or from maternal antibody transferred from an infected parent.

Table 14: Percentage positivity in different category of birds

Category	Seropositive	Seronegative	Total	G total
Clinically ill	15 (51.7%)	14	29	120
Apparently Healthy	51 (56.04%)	40	91	
Broiler birds	36 (41.37%)	51	87	120
Layer bird	30 (90%)	3	33	

Table 15: Comparative blood profile of seropositive versus seronegative birds for CIAV

Parameters	Seropositive (n=66)	Seronegative (n=54)	Overall (n=120)	P value
Haemoglobin (%)**	8.19 ± 0.29	9.67 ± 0.32	8.83 ± 0.23	**0.001**
PCV (%) **	27.32 ± 0.83	30.63 ± 0.76	28.81 ± 0.59	**0.005**
TEC (10^6/µl)	2.29 ± 0.11	2.31 ± 0.09	2.30 ± 0.07	0.874
TWC (10^3/ µl)	18.15 ± 0.73	18.74 ± 0.67	18.42 ± 0.50	0.560
Heterophil (%)	43.36 ± 1.58	42.67 ± 1.72	43.05 ± 1.16	0.766
Lymphocyte (%)	49.77 ± 1.45	50.52 ± 1.71	50.11 ± 1.10	0.738
Monocyte (%)	4.89 ± 0.43	4.48 ± 0.38	4.71 ± 0.29	0.486
Eosinophil (%)	1.53 ± 0.24	1.43 ± 0.29	1.48 ± 0.19	0.782
Basophil (%)	0.53 ± 0.21	0.96 ± 0.20	0.73 ± 0.14	0.138

PCV: packed cell volume, TEC: total erythrocyte count, TWC: total white cells, parameters with ** as superscript differs significantly (P<0.01)

Table 16: Comparative blood profile of clinically-ill (CIL) versus apparently healthy birds

Parameters	Clinically-ill (n=29)	Apparently Healthy (n=91)	Overall (n=120)	P value
Haemoglobin (%)	8.36 ± 0.52	8.97 ± 0.25	8.83 ± 0.23	0.245
PCV (%) **	25.66 ±1.56	29.81 ± 0.56	28.81 ± 0.59	**0.002**
TEC (10^6/µl) **	1.78 ± 0.18	2.47 ± 0.072	2.30 ± 0.074	**0.000**
TWC (10^3/ µl)	18.46 ± 1.11	18.40 ± 0.56	18.42 ± 0.50	0.960
Heterophil (%)	43.90 ± 2.96	42.78 ± 1.21	43.05 ± 1.16	0.681
Lymphocyte (%)	48.76 ± 2.86	50.54 ± 1.14	50.11 ± 1.10	0.492
Monocyte (%)	4.66 ± 0.63	4.73 ± 0.33	4.71 ± 0.29	0.919
Eosinophil (%)	2.00 ± 0.46	1.32 ± 0.20	1.48 ± 0.19	0.118
Basophil (%)	0.48 ± 0.21	0.80 ± 0.18	0.73 ± 0.15	0.348

PCV: packed cell volume, TEC: total erythrocyte count, TWC: total white cells, parameters with ** as superscript differs significantly (P<0.01)

Table 17: Comparative blood profile of CIL birds

S. No.	Parameters	CIL Sero positive	CIL Sero negative	P value
1.	Haemoglobin (%)	7.69±0.93	9.08±0.34	.183
2.	PCV (%)	23.67 ±2.79	27.78±1.09	.192
3.	TEC (10^6/µl)	1.75±0.33	1.80±0.13	NS

PCV: packed cell volume, TEC: total erythrocyte count, CIL: clinically ill, NS: no significance

Table 18: Comparative blood profile of healthy birds

S. No.	Parameters	Sero positive	Sero negative	P value (P<0.01)
1.	Haemoglobin (%)	8.27± 0.27	9.87 ±0.41	.001
2.	PCV (%)	28.39 ±0.65	31.63± 0.91	.004

PCV: packed cell volume, TEC: total erythrocyte count

Table 19: Comparative blood profile of broiler birds (Sero positive vs Sero negative)

S. No.	Parameters	Sero positive	Sero negative	P value (P<0.01)
1.	Haemoglobin (%)	7.92±0.48	9.68± 0.34	0.003
2.	PCV (%)	27.03±1.39	30.65 ± 0.81	0.019
3.	TEC (10^6/µl)	1.92±0.12	2.34 ± 0.09	0.006

PCV: packed cell volume, TEC: total erythrocyte count

Table 20: Comparative blood profile of layer birds (Sero positive vs Sero negative)

S. No.	Parameters	Sero positive	Sero negative	P value
1.	Haemoglobin (%)	8.39±0.28	9.35 ±0.32	NS
2.	PCV (%)	27.67 ±0.77	30.33 ± 0.33	-
3.	TEC (10^6/µl)	1.89 ±0.51	2.73 ±0.17	-

PCV: packed cell volume, TEC: total erythrocyte count, NS: non-significant

CIAV known to have specific tropism towards erythroid stem cells and lymphoid progenitor cells in the bone marrow and thymus respectively. Destruction of erythroid progenitor cells in bone marrow by CIAV cause severe anemia, depletion of granulocytes and thrombocytes in susceptible young birds (Adair 2000, Smyth *et al* 2006). Therefore, along with various other immunosuppressive viral agents, it plays key role in various disease syndrome like haemorrhagic anemia syndrome, infectious/aplastic anemia, anemia-dermatitis syndrome, gangrenous dermatitis and blue wing disease (Bulow and Schat 1997). Various research work showed the specific effect of CIAV on the haemopoietic stem cells.

Vachhani (2005) studied hematological changes in an outbreak of CIA-gangrenous dermatitis syndrome (CIA-GDS) and reported that there was a significant reduction in the PCV, Hb, RBC and WBC counts. The mean concentrations of Hb, PCV, RBC and WBC were 6.8 g percent, 22.77 percent 1.8 X million/cmm and 10.58 X thousand/cmm in affected flocks. Similar findings were also reported by Prajapati (2010)

Bhatt *et al* (2013) observed hypoplasia, anemia and panleukopenia in experimentally infected Specific pathogen free (SPF) chicks with significant ($P<0.05$) decline in Hb, PCV, TEC, TLC, percent lymphocyte count, percent basophil and eosinophil count in all the CIAV infected groups as compared to the healthy birds.

Latheef *et al* (2013) experimentally infected chicks with CIAV and observed a significant change in packed cell volume, haemoglobin level, total white blood cell, red blood cell and percentage lymphocyte counts.

Present study also showed similar changes in haematocrit values of naturally infected seropositive birds with significant ($P<0.01$) changes in PCV, Hb and TEC. Few young birds under the study showed severe decrease in PCV (<6%) and Hb (3%) and they were also found serologically positive for CIAV infection. Among the clinically ill birds, serologically positive showed more pronounced clinical signs of CIA. Among the apparently healthy birds screened, none of them showed any signs of CIA. As CIAV can infect all age groups, the broilers and layers included in the present study were also found positive for antibody to CIAV.

4.1.2.1 Antibody titre profile of birds under the present study

Antibody titre was also calculated as per the manufacturer's instructions (Biocheck CAV antibody ELISA). According to them, an S/P ratio of 0.35 or greater/or titre range of 725 or greater contain CIAV antibodies and were considered as positive in the indirect ELISA protocol. In the present study, an average S/P ratio of 1.22 and 0.11 were observed among the seropositive and seronegative birds and an average antibody titre of 2873 and 183 were observed among the seropositive and seronegative birds. The titre values of the birds under 0-10 weeks were having lowest (909) and highest (5000) range of antibody titre. The birds of age group greater than 10 shown to have moderate antibody titre (Table 21). Since the samples were from unvaccinated birds, the present study assumes that the birds were seroconverted to CIAV from natural infection and moderate to high level of antibody titre may be due to the persistence of virus in apparently healthy birds.

An important finding in the present study was the presence of anti-CIAV antibody in apparently healthy chicken along with DNA of CIAV in seropositive birds. Out of 91 apparently healthy birds screened by indirect ELISA, 51 (56.04%) were found positive for anti-CIAV antibody while remaining 40 birds were found negative. All age groups under the study showed presence of antibody to chicken

anemia virus. 34.21% in 0-5 week, 57.14% in 5-10 weeks and 100% in all birds greater than 10 weeks were found positive for anti-CIAV antibody. The birds under the younger age (<10 weeks) group had higher antibody titre with an average titre value of 3413.76 than older one which showed an average titre value of 2198.31. Owoade *et al* (2004) suggest that adult birds may seroconverted without any obvious symptoms which was in agreement with our study in apparently healthy birds. According to BioChek CAV antibody ELISA guidelines, an anti-CAV antibody titer ranges of no protection, moderate protection and protection are 724, 724-2295 and >2296, respectively (CAV 2016, Chansiripornchai 2016). Therefore, the birds which had no antibody titre or lower antibody titre (<724) may be under risk to get infected from natural infection. In the present study, the birds under 2-7 weeks of age were found negative for anti-CIAV antibody. As CIAV can cause severe disease in young birds of 2-3 weeks of age, most of the birds which were found negative are under risk of getting infection and further development of clinical disease.

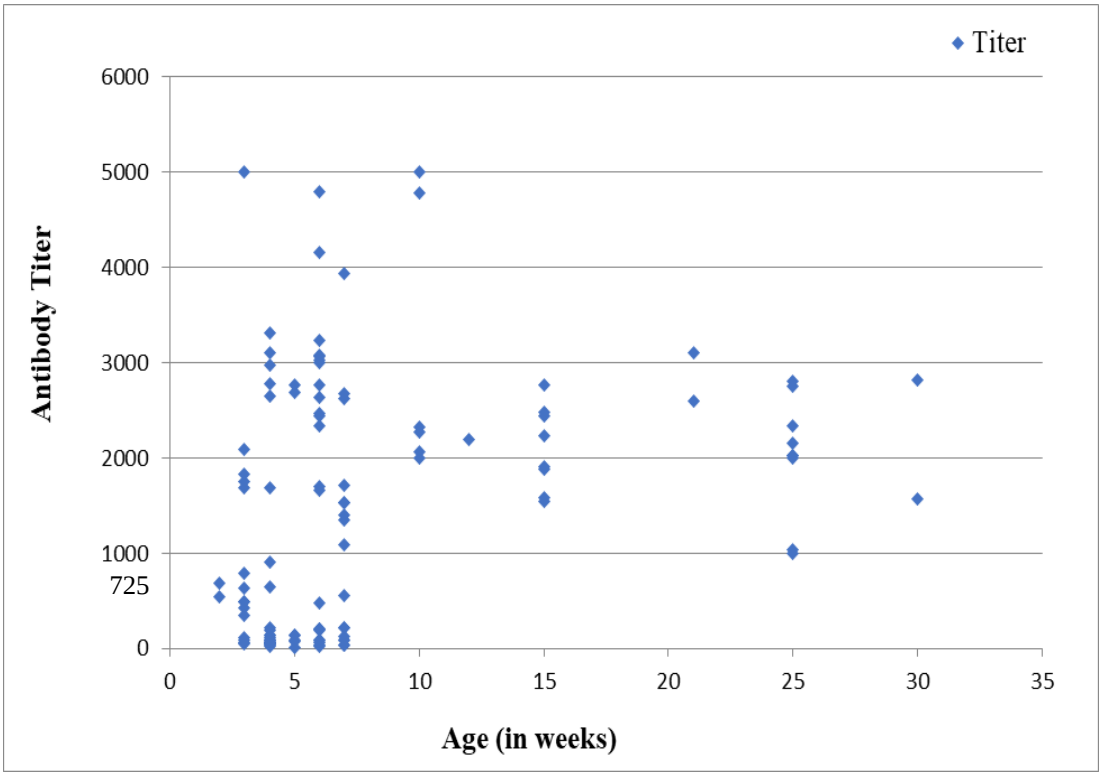
Canal *et al* (2004) reported that breeder hens subject to natural CIAV infection show great variation in anti-CIAV antibody titers and absence or lower antibody in them indicate that there was some potential to transmit the virus vertically. According to their findings, all vaccinated hens had antibody titer to CIAV capable of conferring protection to chicks against clinically apparent disease.

Mahzounieh *et al* (2005) conducted serological study in chicken flocks of all age group and the rates of antibody-positive chickens among flocks ranged from 20% to 100%. An anti-CIAV antibody titres minimum of 0 and a maximum of 72850 were observed among the flocks in Sharekord area, Iran

Khoshkhoo *et al* (2011) compared CAV antibody titers in vaccinated and naturally infected broiler breeder flocks. Based on their study, an antibody titre of 5000 and above found to be protective which can be achieved by vaccinating the breeder flock with live vaccine. The administration of live CIAV vaccine appeared to cause an increase in antibody titre and further maintained the homogeneity of antibody titer in progenies of vaccinated breeder compared to naturally infected breeder.

Difference in values of antibody titre for protection is due to difference in ELISA kit recommended by manufactures which were used in various studies.

Table 21: Antibody titre of the birds under study



4.1.3 Comparison of serology with molecular study

Serological results from the present study indicates high incidence of CIAV among poultry flocks. So that, to confirm the presence of virus in the seropositive birds, the seropositive blood samples were further screened for CIAV genome by PCR method. VP3 gene were amplified as it is the highly conserved region of CIAV genome. A positive control from CIAV vaccine strain was also included in the PCR run. The primers employed for amplification of VP3 gene could amplify CIAV VP3 sequence to produce 364 bases as expected. The negative control (NTC) did not show any amplification (Figure 7).

Out of the 66 seropositive birds 49 (74.24%) were amplified VP3 gene of CIAV. Among the 15 seropositive clinically ill birds, 12 (80%) were found positive for CIAV gene and among 51 seropositive apparently healthy bird, 37 (72.54%) were found positive for CIAV gene. This finding further confirms the presence of CIAV among the chicken flocks. The haematological parameters of these birds were also

compared with molecular study which showed marginal significance in total WBC (P value <0.06) count in PCR positive birds (Table 22).

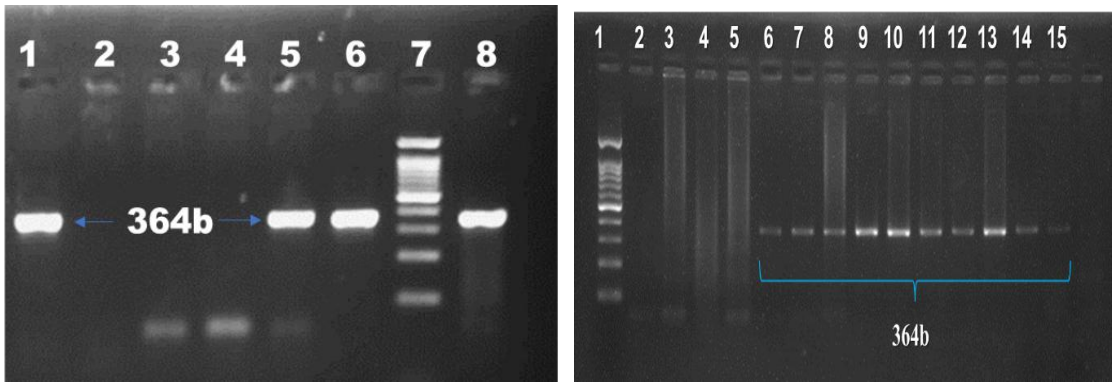
Table 22: Comparative blood profile of PCR positive versus negative birds for CIAV

Parameters	PCR +ve (n=49)	PCR -ve (n=17)	Overall (n=66)	P value
Haemoglobin (%)	8.09 ± 0.34	8.18 ± 0.56	8.11 ± 0.29	0.899
PCV (%)	27.35 ± 0.96	26.94 ± 1.68	27.24 ± 0.83	0.832
TEC (10^6/µl)	2.28 ± 0.14	2.31 ± 0.20	2.29 ± 0.11	0.910
TWC (10^3/ µl)	17.36 ± 0.91	20.50 ± 0.90	18.16 ± 0.73	0.060
Heterophil (%)	42.55 ±1.75	46.12 ±3.42	43.47 ±1.57	0.323
Lymphocyte (%)	50.41 ±1.61	47.35 ±3.16	49.62 ±1.44	0.359
Monocyte (%)	4.69 ± 0.51	5.47 ± 0.85	4.89 ± 0.43	0.438
Eosinophil (%)	1.57 ± 0.28	1.41 ± 0.47	1.53 ± 0.24	0.774
Basophil (%)	0.71 ± 0.28	0.18 ± 0.10	0.58 ± 0.21	0.272

PCV: packed cell volume, TEC: total erythrocyte count, TWC: total white cells

Molecular analysis of CIAV genome from the blood sample was done by various researchers. Even though, there was seroconversion among chicken flocks, the viral DNA still can be vertically transmitted to progeny. (Cardona *et al* 2000, Hussein *et al* 2002, Miller *et al* 2003, Brentano *et al* 2005, Li *et al* 2016).

Tham and Stanislawek (1992) conducted PCR assay on serum samples from disease-free chickens and they detected CAV DNA in two out of 37 samples. Novak and Ragland (1997) demonstrated CIAV DNA in circulating blood cells by *In siut* hybridization of lymphocytes from peripheral blood smears. Hussein *et al* (2002) reported that a 418base of CIAV genome sequence was amplified from the DNA extracted from the blood samples. Oluwayelu (2008) could amplify VP1 gene sequence from serum samples of back yard poultry and Li *et al* (2016) reported PCR amplification of CIAV DNA from SPF chicken blood. Present study also shows similar results and the presence CIAV DNA in the seropositive blood sample is a clear indication of CIAV infection in the flocks.



(A) **(B)**

Fig. 7: Agarose gel electrophoresis of PCR amplifying VP3 gene of CIAV from seropositive blood samples

A) Lane 1: Positive control; Lane 2: Non-template control (NTC); Lane 3 to 4: Negative samples; Lane 5,6 and 8: Positive samples (364bases); Lane 7: Marker (100bp)

B) Lane 1: Marker (100bp); Lane 2-5: Negative samples; Lane 6-15: Positive samples

4.1.4 Sequence analysis

From the blood sample positive for CIAV DNA (VP3), six highly positive purified DNA samples (by DNA concentration) were sent for sanger's sequencing. The VP3 gene sequences obtained were analyzed first by BLAST (NCBI) analysis which showed high similarity with other CIAV VP3 gene sequences available in the Databank. Further sequence was analyzed by DNASTAR(ClustalW) (Chenna *et al* 2003) and MEGA.7 softwares.

The VP3 gene of CIAV is composed of 364 bases. The sequence of the six sample from the present study were aligned with 12 published VP3 gene sequences retrieved from GenBank (NCBI) (Table 23). Nucleotide alignment report shows high sequence similarity with reference sequences. Multiple sequence alignment of all the six sequence from the present study with reference sequences revealed that highest percentage identity with sequences from Punjab, Namakal, Haryana, Maharashtra and Ludhiana (99.7%) and lowest with Tamil Nadu (96.7%) (Figure 8). The nucleotide identity among the six isolates from present study showed 100% similarity except one sequence (Patiala) which was 99.7%.

4.1.5 Phylogenetic analysis

Phylogenetic analysis to deduce the evolutionary relationship among the virus isolate was carried out by using MEGA7 software and ClustalW algorithm. Maximum likelihood (ML) tree (based on retrieved reference nucleotide sequences) was constructed using Jukes-Cantor model (Figure 9). On the basis of complete VP3 gene sequence, the six isolates from the present study did not form any distinct Clad/cluster with the reference isolates. The isolates from Maharashtra and Punjab were branched at close proximity to the isolates from present study. Isolate from Tamil Nadu and Haryana branched distantly from Punjab isolates. It also closely related to reference isolates from various countries.

Since no specific clad or cluster formation was observed between the isolates from the present study and its close relation with reference CIAV isolates, it is assumed that the DNA sequence obtained from the seropositive blood sample is of CIAV. Therefore, PCR and sequence analysis of the isolates from this study clearly indicate that CIAV is circulating among chicken flocks of Punjab state and isolates are closely related to other reported CIAV isolates from various parts of India and the world.

Table 23: Detail of reference sequences and sequences from the present study used for molecular analysis VP3 gene of CIAV from blood samples

S. No.	ID of Isolates	Accession Number	Geographical location
1.	*Brazil*	MG846491.1	Brazil
2.	*Malaysia*	AF390038.1	Malaysia
3.	*USA*	AF313470.1	USA
4.	*Maharashtra*	EF159945.1	Maharashtra
5.	*LDH*	MG720298.1	Punjab
6.	*PUNJ-FLK*	MH269370.1	Punjab (Present study)
7.	*LDH*	MG720297.1	Punjab
8.	*Argentina*	KJ872513.1	Argentina
9.	*BL64*	MK385602	Punjab (Present study)
10.	*Iran*	KT276305.1	Iran
11.	*BL31*	MK385599	Punjab (Present study)
12.	*Taiwan*	KJ728826.1	Taiwan
13.	*BL30*	MK385598	Punjab (Present study)
14.	*Taiwan*	MG827098.1	Taiwan
15.	*China*	KU645522.1	China
16.	*BL17*	MK385597	Punjab (Present study)
17.	*New York*	L14767.1:	New York
18.	*BL57*	MK385600	Punjab (Present study)
19.	*CAV-EG*	MH001568	China
20.	*Taiwan*	KY888938.	Taiwan
21.	*China*	KU050677.1	China
22.	*BL63*	MK385601	Punjab (Present study)
23.	*LDH*	MG720296.1	Punjab
24.	*Haryana-Karnal*	EF189153.1	Haryana
25.	*UP*	KF716161.1	UP
26.	*Egypt*	MH001557.1	Egypt
27.	*UK*	U66304.1	UK
28.	*cux1*	M55918.1	USA
29.	*CAVNAMKS6*	KP129275.1	Tamil Nadu

MegAlign - [Pair Distances of percentage id.meg ClustalW (Slow/Accurate, IUB)]

File Edit Align View Options Net Search Help

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1		100.0	100.0	100.0	100.0	99.7	99.7	98.1	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	1
2	0.0		100.0	100.0	100.0	99.7	99.7	98.1	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	2
3	0.0	0.0		100.0	100.0	99.7	99.7	98.1	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	3
4	0.0	0.0	0.0		100.0	99.7	99.7	98.1	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	4
5	0.0	0.0	0.0	0.0		99.7	99.7	98.1	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	5
6	0.3	0.3	0.3	0.3	0.3		99.5	97.8	99.5	99.5	99.5	96.7	99.2	98.9	99.2	99.5	99.2	99.5	6
7	0.3	0.3	0.3	0.3	0.3	0.6		98.4	99.7	100.0	100.0	97.0	99.7	99.5	99.7	100.0	100.0	100.0	7
8	1.9	1.9	1.9	1.9	1.9	2.2	1.7		98.1	98.4	98.4	95.4	98.1	97.8	98.1	98.4	98.4	98.4	8
9	0.3	0.3	0.3	0.3	0.3	0.6	0.3	1.9		99.7	99.7	96.7	99.5	99.2	99.5	99.7	99.7	99.7	9
10	0.3	0.3	0.3	0.3	0.3	0.6	0.0	1.7	0.3		100.0	97.0	99.7	99.5	99.7	100.0	100.0	100.0	10
11	0.3	0.3	0.3	0.3	0.3	0.6	0.0	1.7	0.3	0.0		97.0	99.7	99.5	99.7	100.0	100.0	100.0	11
12	2.5	2.5	2.5	2.5	2.5	2.8	2.5	4.2	2.8	2.5	2.5		97.0	97.8	97.0	97.3	97.3	97.3	12
13	0.6	0.6	0.6	0.6	0.6	0.8	0.3	1.9	0.5	0.3	0.3	2.8		99.2	99.5	99.7	99.7	99.7	13
14	0.8	0.8	0.8	0.8	0.8	1.1	0.5	2.2	0.8	0.5	0.5	1.9	0.8		99.2	99.5	99.5	99.5	14
15	0.6	0.6	0.6	0.6	0.6	0.8	0.3	1.9	0.5	0.3	0.3	2.8	0.5	0.8		99.7	99.7	99.7	15
16	0.3	0.3	0.3	0.3	0.3	0.6	0.0	1.7	0.3	0.0	0.0	2.5	0.3	0.5	0.3		100.0	100.0	16
17	0.3	0.3	0.3	0.3	0.3	0.6	0.0	1.7	0.3	0.0	0.0	2.5	0.3	0.6	0.3	0.0		100.0	17
18	0.3	0.3	0.3	0.3	0.3	0.6	0.0	1.7	0.3	0.0	0.0	2.5	0.3	0.5	0.3	0.0	0.0		18
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

1 BL64PATI
2 BL17SANGU
3 BL30LDH
4 BL31LDH
5 BL57PATI
6 BL63PATI
7 MH269370.1 Punja.
8 AF520789.1 MP
9 AF520788.1 IND NAM VP3
10 EF159944.1 IND HR VP3
11 EF159945.1 IND MAH VP3
12 FJ883790.1 IND TAM VP3
13 KF716161.1 IND UP VP3
14 KP129279.1 IND NAM VP3
15 MG720296.1 IND LDH VP3
16 MG720297.1 IND LDH VP3
17 MG720298.1 IND LDH VP3
18 PUNJ-FLK VP3

Fig. 8: **Distance matrix of VP3 gene nucleotide sequences of CIAV from seropositive birds. Percentage identity/divergence matrix generated by multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of full length VP3 gene nucleic acid sequences. Upper triangle represents the percentage of identity between two viruses. Lower triangle represents the percentage of divergence between two viruses. CIAV isolates from the present study showing 96.7% to 99.7% identity with reference sequences and 100% similarity among them**

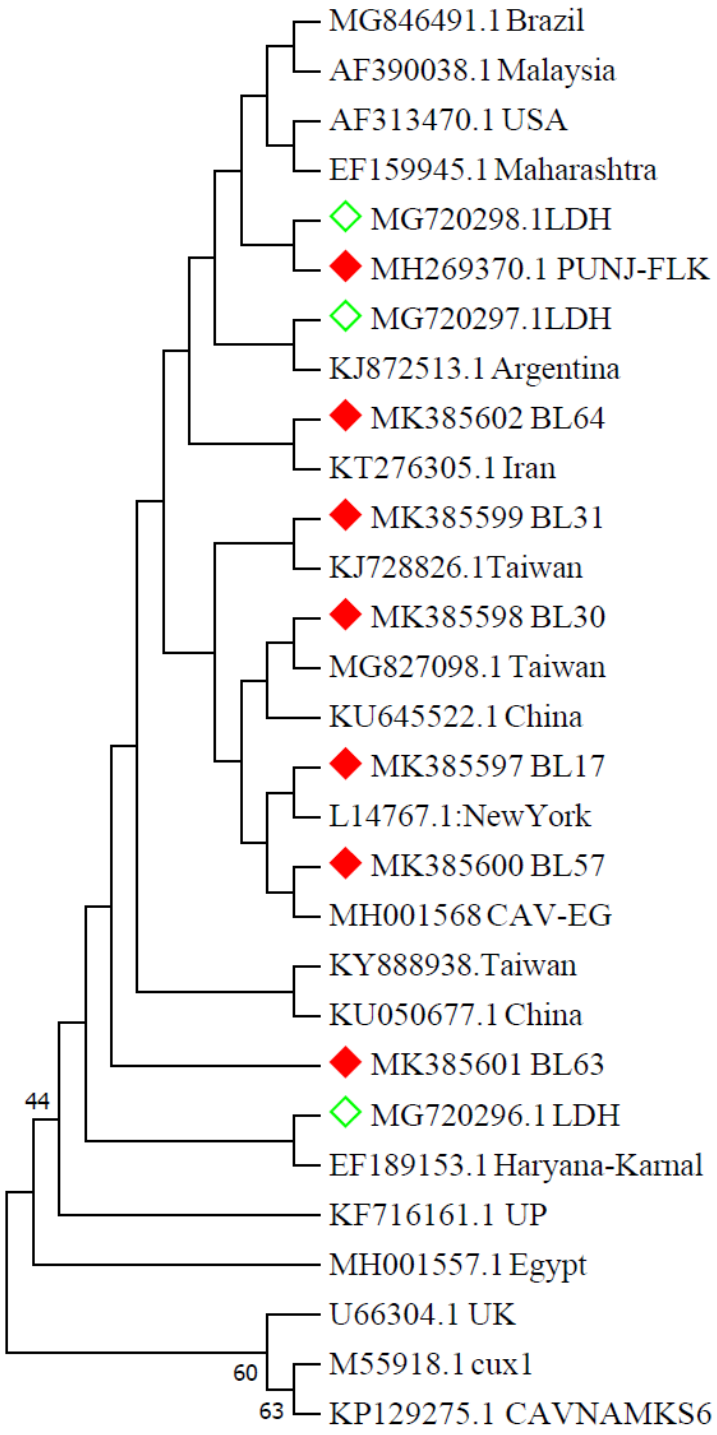


Fig. 9: Molecular Phylogenetic analysis of VP3 gene by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Red boxes indicate isolates from the present study. Punj-FLK, BL 17, 30, 31, 57, 63and 64 formed single group along with other Indian and world isolates.

4.2 Detection of Chicken Infectious Anemia Virus from tissue samples

4.2.1 Anamnesis and clinical signs of the dead birds presented for necropsy examination

For isolation of CIAV from the chicken flocks, various lymphoid tissues were collected from dead birds suspected of CIAV infection. The study was carried out on 65 dead birds from 16 different chicken flocks covering 11 different geographical areas of Punjab (Table 24). Birds inclusion in the present study was based on history of one or more CIA specific clinical signs in young dead birds and/or based on necropsy findings. Clinical signs reported were droopiness, sudden death and high mortality rate among chicken flocks. Lesions observed in the necropsy finding includes paleness of the carcass, haemorrhage in the subcutaneous tissues, muscle serosa and proventriculus, Bursa of fabricius, pale bone marrow and liver, gangrenous dermatitis of the wing *etc* (Figure 10). For molecular detection and isolation of field CIAV, a total of 350 tissue samples (Thymus, bone marrow, Liver, spleen and bursa of fabricius) from 65 dead birds were screened by PCR and further isolated by cell culture method.

Since its first report during last decades of 20^{th} century, various studies were conducted to characterize CIAV both biologically and molecularly. According to the various reports, CIAV is highly prevalent among chicken flocks and possesses potential to become a single cause of havoc for 22^{nd} century poultry sector. CIA- the disease is characterized by loss of weight gain and poor performance, aplasia of bone marrow and severe anemia, generalized lymphoid atrophy with concomitant immunosuppression (Adair 2000, Gowthaman *et al* 2014, Krishan *et al* 2015). In the present study, birds also showed one or more specific clinical signs of the disease. Hemorrhage in the subcutaneous tissue, serosa of muscles and pale bone marrow were consistent finding in young birds.

4.2.2 DNA extraction and PCR optimization

DNA was extracted from each tissue samples and quantified by Nanodrop spectrophotometry. The concentration of DNA eluted ranged from 0.15µg – 0.85 µg. the concentration of DNA was optimized to 0.45-0.5 µg/µl in PCR protocol.

Table 24: Molecular detection of CIAV in tissue samples collected from various farms

S. No.	Geographical area	Presence of CIAV in farms			No of birds screened		
		Total farms	Positive	Negative	Total birds	Positive	Negative
1.	Fazilka	2	2	-	2	2	-
2.	Ludhiana	3	2	1	27	25	2
3.	Moga	3	1	2	9	5	4
4.	Ferozepur	1	1	-	2	1	1
5.	Hoshiarpur	1	-	1	8	-	8
6.	Mansa	1	1	-	3	1	2
7.	Barnala	1	1	-	2	1	1
8.	Jalandhar	1	-	1	2	-	2
9.	Mohali	2	2	-	6	3	3
10.	Sangrur	1	1	-	4	1	3
	Total	**16**	**11**	**5**	**65**	**39**	**26**

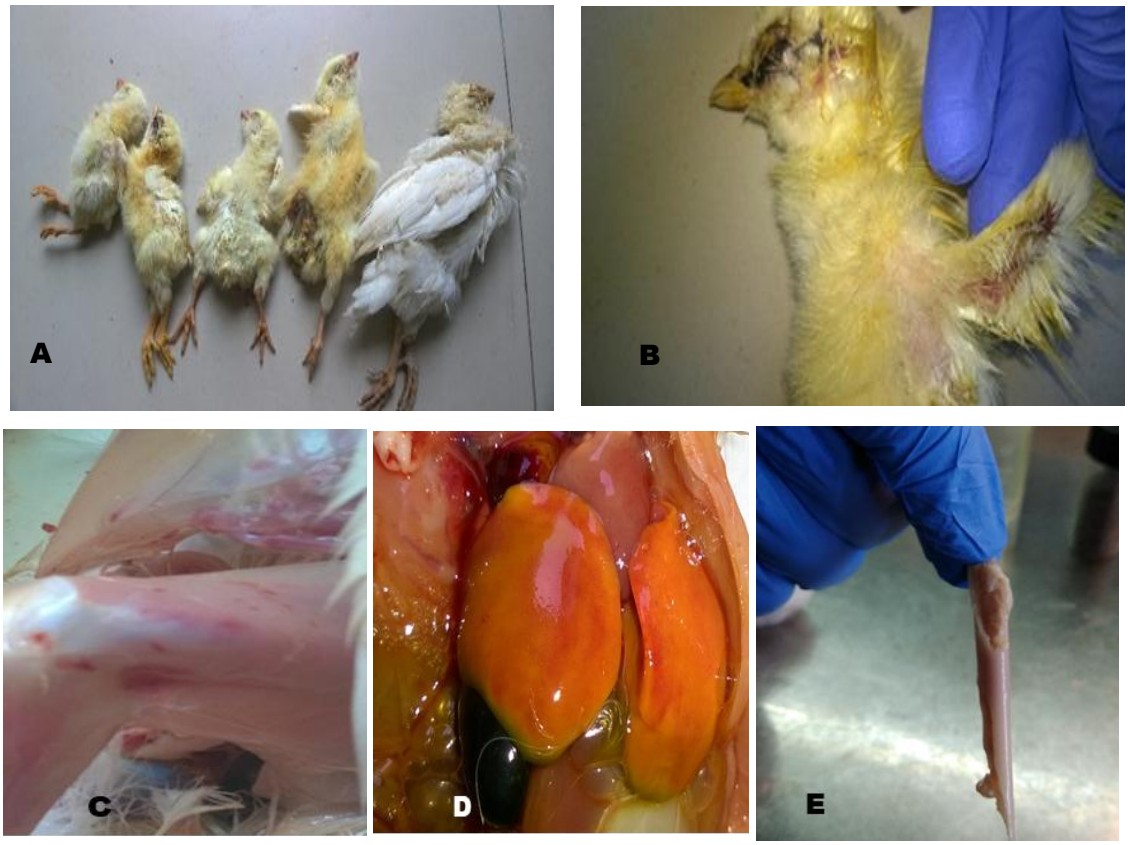


Fig. 10: Clinical signs and necropsy findings in CIAV suspected birds.

A) Mortality among young chick

B) Gangrenous dermatitis

C) Haemorrhages in serosa of muscle

D)Pale swollen Liver

E) Pale bone marrow

4.2.3 Detection of CIAV by PCR

The DNA of CIAV was detected by VP1 specific PCR from the various tissue samples. VP1 gene specific primer yielded expected amplified products of 1350 bases (Figure 11). No band was observed in the non-template (NTC). The samples positive for VP1 gene were also screened for VP2 and VP3 gene which also yielded expected PCR amplicon size of 652 bases and 364 bases, respectively (Figure 12 and 13).

In the present study, out of 65 birds 39 (60%) were found positive for CIAV which indicates that, the birds were infected with CIAV. A total of 16 farms were screened for the presence of CIAV, out of it, 11 (69%) were found positive (Table 24) for CIAV. None of the birds under the study were vaccinated for CIAV. Therefore, the detected virus may be a wild type circulating among bird population. Among tissues, thymus was found to be having highest percentage of the virus. Out of 50 thymus tissues screened, 33 (66%) were found positive for CIAV and least was observed in liver and spleen with 28 each (56%) (Table 25).

CIAV can infect all age group, sex, breed, layers and broilers, breeders and it is ubiquitous in nature. Various age group ranging from less <7days to >60 days were also included in the present study. Among this groups, chicks with age group of 0-20 days (92.86%) had highest incidence of infection and disease (Table 26). It is primarily a disease of young chicks of <3 weeks of age but virus can persistently infect older birds also. The current study also observed similar incidence in age group of <3 weeks and similar findings were also reported by various other researchers (Yuasa *et al* 1979, Wani *et al* 2013).

Table 25: Detection of CIAV from different lymphoid organs

S. No.	Tissues screened for CIAV	PCR		Total
		Positive	Negative	
1.	Thymus	33	17	50
2.	Liver	28	22	50
3.	Bone marrow	31	19	50
4.	Spleen	28	22	50
5.	Bursa of fabricius	30	20	50
6.	Total	150	100	250

Table 26: Detection of CIAV based on the age group

S. No.	Age group (in Days)	Positive	Negative	Total
1.	0-20	13	1	14
2.	21-40	7	6	13
3.	41-60	18	18	36
4.	>60	1	1	2

The VP1 gene specific primers binds at the position of 853bases to 870bases and 2199bases to 2202bases amplifying 1350bases PCR products. VP2 gene specific primers binds at 380-390bases and 1011 -1030bases which yielded 652bases amplified PCR products. The most conserved gene in CIAV genome is VP3 gene and the specific primer for VP3 binds at the position of 486 – 507bases and 827 -848bases which amplified 364bases PCR product. The PCR amplification of various important gene with the same amplicon size was also reported by Wani *et al* (2013). Among the three gene screened, highly variable region of CIAV genome is residing in the VP1 gene and it transcribe into highly immunogenic protein (Todd *et al*1990) which is the base of capsid in virus particle. Co-expression of VP1 and VP2 protein involved in production of antigenicity and induction of more potent neutralizing antibody (Noteborn *et al* 1998, Lee *et al* 2011) production.

Nogueira *et al* (2005) designed PCR assay for amplifying highly conserved VP3 gene and 5' end of VP1 gene from CIAV genome. The results were further confirmed with restriction enzyme analysis and partial sequence analysis.

Hailemariam *et al* (2008) amplified CIAV specific DNA by targeting VP1 gene. They amplified 498 bases of VP1 coding region of genome of CIAV. Kim *et al* (2010) sequenced CIAV genome from various flocks of breeder and commercial chickens by amplifying 675 bases of VP1 gene.

Vaziry *et al* (2011) targeted VP3 gene to quantify the viral load in various lymphoid organs of experimentally infected chicks by nested PCR. Hiremath *et al* (2013) detected CIAV by PCR in tissue samples collected from poultry flocks in Gujarat, India. They targeted all the three important viral genes *vis* VP1, VP2 and VP3. The amplification yielded each gene specific product of 1390 bases, 713 bases and 367 bases for VP1, VP2 and VP3 genes, respectively

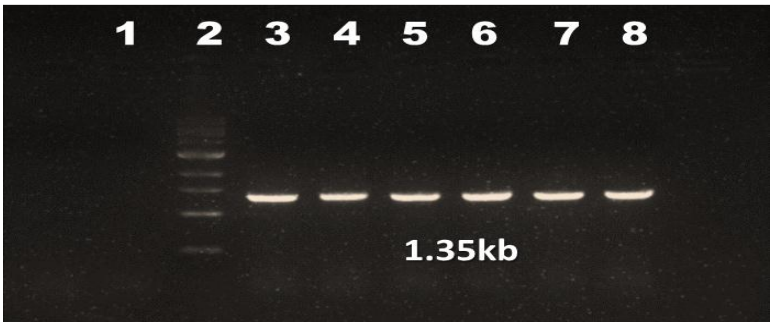


Fig. 11: Agarose gel electrophoresis of PCR amplifying VP1 gene of CIAV field isolates

Lane 1: Non-template control (NTC); Lane 2: Marker (1Kb); Lane 3: Positive control; Lane 4-8: samples (1350bases)

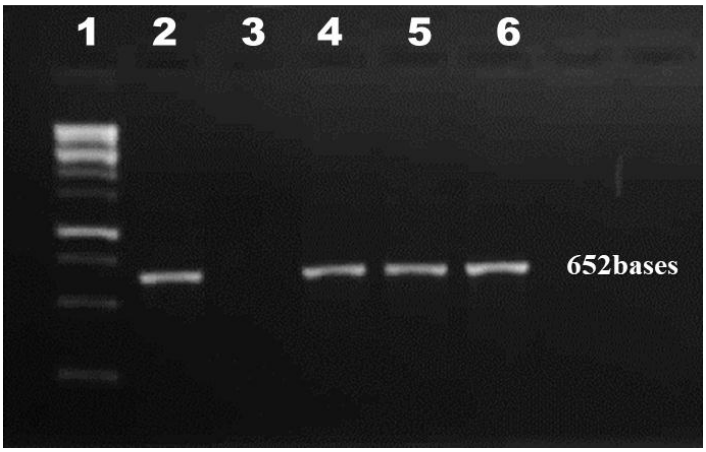


Fig. 12: Agarose gel electrophoresis of PCR amplifying VP2 gene of CIAV field isolates

Lane 1: Marker (1kb); Lane 2: Positive control; Lane 3: Non-template control (NTC); Lane 4 to 6: Field samples (652b)

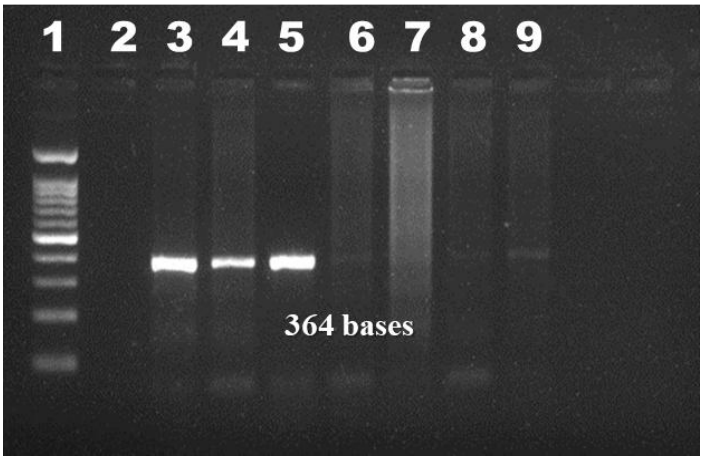


Fig. 13: Agarose gel electrophoresis of PCR amplifying VP3 gene of CIAV field isolates

Lane 1: Marker (100bp); Lane 2: Non-template control (NTC); Lane 3: Positive control; Lane 4-9: Field samples

Wani *et al* (2013) developed PCR amplifying all the three viral genes VP1 VP2 and VP3 for the molecular epidemiological study. The developed PCR test were highly specific and sensitive for the detection of CIAV DNA in the field samples. The specific primers amplified the entire coding region of VP1, VP2 and VP3 gene which encompasses 1350bases, 651bases and 363bases, respectively.

Gowthaman *et al* (2014) amplified 419bases of VP2 gene from commercial poultry flocks with a history of respiratory disease complex from four different states of India and reported that CIAV specific DNA was detected in 30 out of 32 clinical cases studied. Subclinical infections, in concurrence with other respiratory pathogens may significantly decreased the flock performance, increased the incidence of vaccine failures and other immunosuppressive and respiratory pathogens.

Our study also agrees with previous reports which amplified all the three important viral genes i.e. VP1 VP2 and VP3.

4.2.4 Sequencing analysis of VP1, VP2 and VP3 genes

From the PCR positive tissue sample, highly positive (by DNA concentration) sample was selected for sequencing and purified DNA samples were send for sanger's method of sequencing. The VP1 gene sequence obtained in the present study consist of 1300 bases. Initial BLAST **a**nalysis of the VP1 gene sequences showed high similarity with the reference sequences of CIAV isolates available (Table 27) in the GenBank (NCBI).

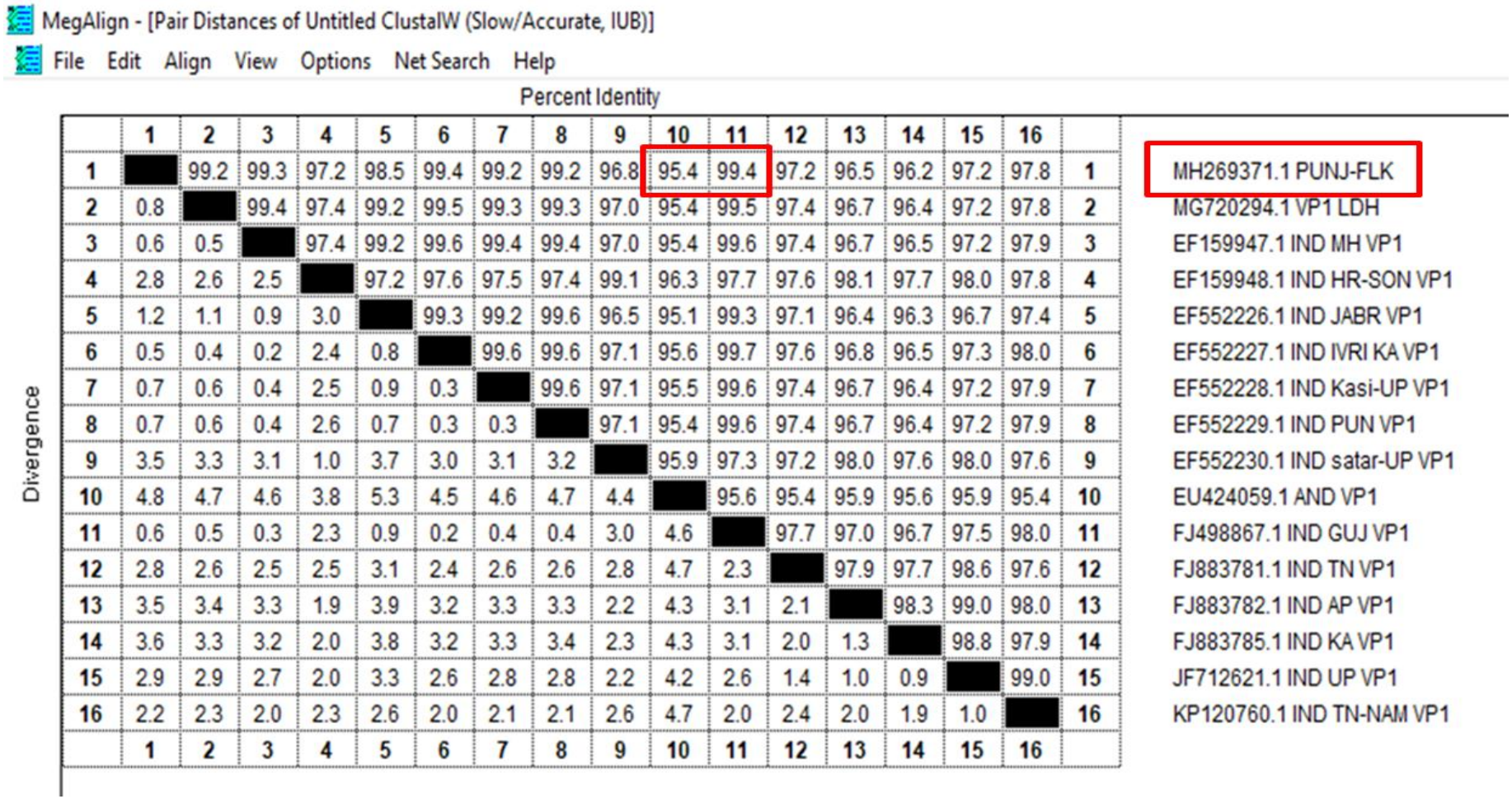
Further, sequences were analyzed by DNASTAR and MEGA.7 software. VP1 gene showed 99.4% identity with isolates from Gujrat, UP and lowest identity of 95.4% with isolates from Anand (Figure 14). There were many points mutations could be observed in VP1 gene as it is the most variable region in the CIAV genome. Also, deduced alignment of the amino acid sequence shows some conservative mutation (L to I and S-A) in VP1 viral protein (Figure 15).

Various researchers worked on CIAV by targeting the VP1gene and genetically characterized. They suggested that amino acid Q (glutamine) at position 394 in VP1 gene may be a major determinant of pathogenicity and amino acid H in the same position may leads to law pathogenicity (Natesan *et al* 2006, Kim *et al* 2010, Hiremath *et al* 2013, Olszewska-Tomczyk *et al* 2016, Erfan *et al* 2018). The isolate from the present study had amino acid K at 139, E at144 and Q at 394 position. Some other variation in amino acid observed are L at 135, S at 287, I at 376 and S at 413 (Figure 15).

Table 27: Detail of reference sequences and sequence from the present study used for molecular analysis VP1

S. No.	ID of Isolates	Accession Number	Geographical location
1.	*Jabalpur*	EF552226.1	Jabalpur
2.	*Kasipur*	EF552228.1	U.P.
3.	*Karnataka*	EF552227.1	Karnataka
4.	*Maharashtra*	EF159947.1	Maharashtra
5.	*PUNJ-LDH*	MG720294.1	Punjab
6.	*PUNJ-FLK*	MH269371.1	Punjab-Fazilka Present Study
7.	*Gujarat*	FJ498867.1	Gujarat
8.	*CAVNAMKS7*	KP120760.1	Tamil Nadu
9.	*Satara*	EF552230.1	U.P.
10.	*Haryana-Sonipat*	EF159948.1	Haryana
11.	*NDTN06*	FJ883781.1	Tamil Nadu
12.	*IND AP 07*	FJ883782.1	Andhra Pradesh
13.	*India VP1 gene*	JF712621.1	

The VP2 and VP3 gene sequences obtained in the present study consist of 652 bases and 363 bases, respectively. Initial BLAST analysis of VP2 and VP3 shows high sequence similarity with reference sequences (Table 28 and Table 29). When compared with VP1 gene, VP2 and VP3 genes are highly conserved in CIAV genome and the sequence obtained from the present study did not show any nucleotide substitution (Figure 16 and 17). Wang *et al* (2006) targeted VP2 gene of CIAV genome extracted from chicken liver tissue by PCR method. They successfully amplified VP2 gene and cloned to express recombinant viral protein.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		99.2	99.3	97.2	98.5	99.4	99.2	99.2	96.8	95.4	99.4	97.2	96.5	96.2	97.2	97.8	**1**
2	0.8		99.4	97.4	99.2	99.5	99.3	99.3	97.0	95.4	99.5	97.4	96.7	96.4	97.2	97.8	**2**
3	0.6	0.5		97.4	99.2	99.6	99.4	99.4	97.0	95.4	99.6	97.4	96.7	96.5	97.2	97.9	**3**
4	2.8	2.6	2.5		97.2	97.6	97.5	97.4	99.1	96.3	97.7	97.6	98.1	97.7	98.0	97.8	**4**
5	1.2	1.1	0.9	3.0		99.3	99.2	99.6	96.5	95.1	99.3	97.1	96.4	96.3	96.7	97.4	**5**
6	0.5	0.4	0.2	2.4	0.8		99.6	99.6	97.1	95.6	99.7	97.6	96.8	96.5	97.3	98.0	**6**
7	0.7	0.6	0.4	2.5	0.9	0.3		99.6	97.1	95.5	99.6	97.4	96.7	96.4	97.2	97.9	**7**
8	0.7	0.6	0.4	2.6	0.7	0.3	0.3		97.1	95.4	99.6	97.4	96.7	96.4	97.2	97.9	**8**
9	3.5	3.3	3.1	1.0	3.7	3.0	3.1	3.2		95.9	97.3	97.2	98.0	97.6	98.0	97.6	**9**
10	4.8	4.7	4.6	3.8	5.3	4.5	4.6	4.7	4.4		95.6	95.4	95.9	95.6	95.9	95.4	**10**
11	0.6	0.5	0.3	2.3	0.9	0.2	0.4	0.4	3.0	4.6		97.7	97.0	96.7	97.5	98.0	**11**
12	2.8	2.6	2.5	2.5	3.1	2.4	2.6	2.6	2.8	4.7	2.3		97.9	97.7	98.6	97.6	**12**
13	3.5	3.4	3.3	1.9	3.9	3.2	3.3	3.3	2.2	4.3	3.1	2.1		98.3	99.0	98.0	**13**
14	3.6	3.3	3.2	2.0	3.8	3.2	3.3	3.4	2.3	4.3	3.1	2.0	1.3		98.8	97.9	**14**
15	2.9	2.9	2.7	2.0	3.3	2.6	2.8	2.8	2.2	4.2	2.6	1.4	1.0	0.9		99.0	**15**
16	2.2	2.3	2.0	2.3	2.6	2.0	2.1	2.1	2.6	4.7	2.0	2.4	2.0	1.9	1.0		**16**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	

1 MH269371.1 PUNJ-FLK
2 MG720294.1 VP1 LDH
3 EF159947.1 IND MH VP1
4 EF159948.1 IND HR-SON VP1
5 EF552226.1 IND JABR VP1
6 EF552227.1 IND IVRI KA VP1
7 EF552228.1 IND Kasi-UP VP1
8 EF552229.1 IND PUN VP1
9 EF552230.1 IND satar-UP VP1
10 EU424059.1 AND VP1
11 FJ498867.1 IND GUJ VP1
12 FJ883781.1 IND TN VP1
13 FJ883782.1 IND AP VP1
14 FJ883785.1 IND KA VP1
15 JF712621.1 IND UP VP1
16 KP120760.1 IND TN-NAM VP1

Fig. 14: **Distance matrix of VP1 gene nucleotide sequences of CIAV. Percentage identity/divergence matrix generated by multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of partial VP1 gene nucleic acid sequences. Upper triangle represents the percentage of identity between two viruses. Lower triangle represents the percentage of divergence between two viruses. CIAV isolates from the present study showing 95.4% to 99.4% identity with reference sequences**

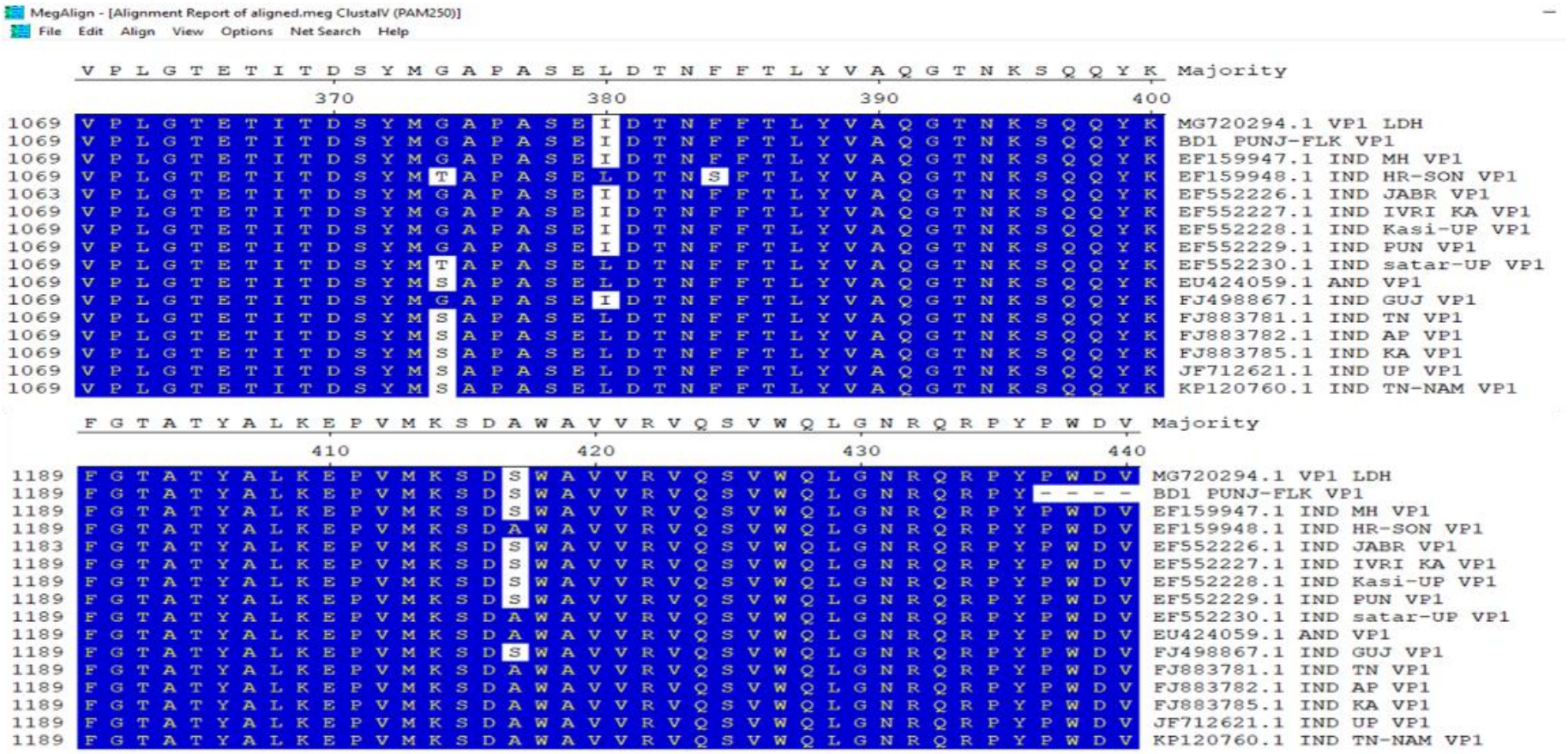


Fig. 15: Multiple sequence alignment of deduced amino acid of VP1 protein using ClustalW algorithm (MegAlign, DNASTAR). L to I (380) and A to S (417) Substitution

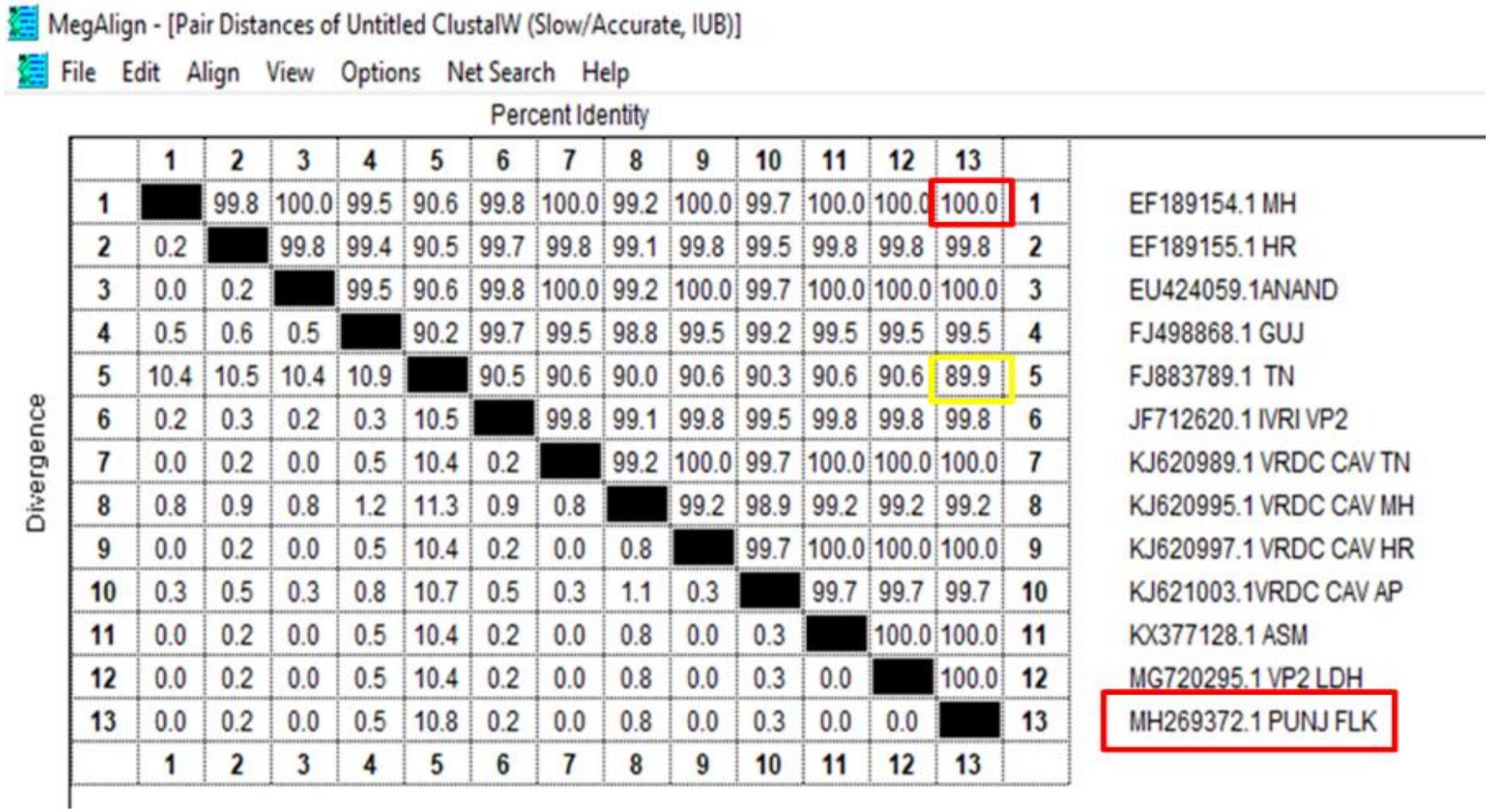
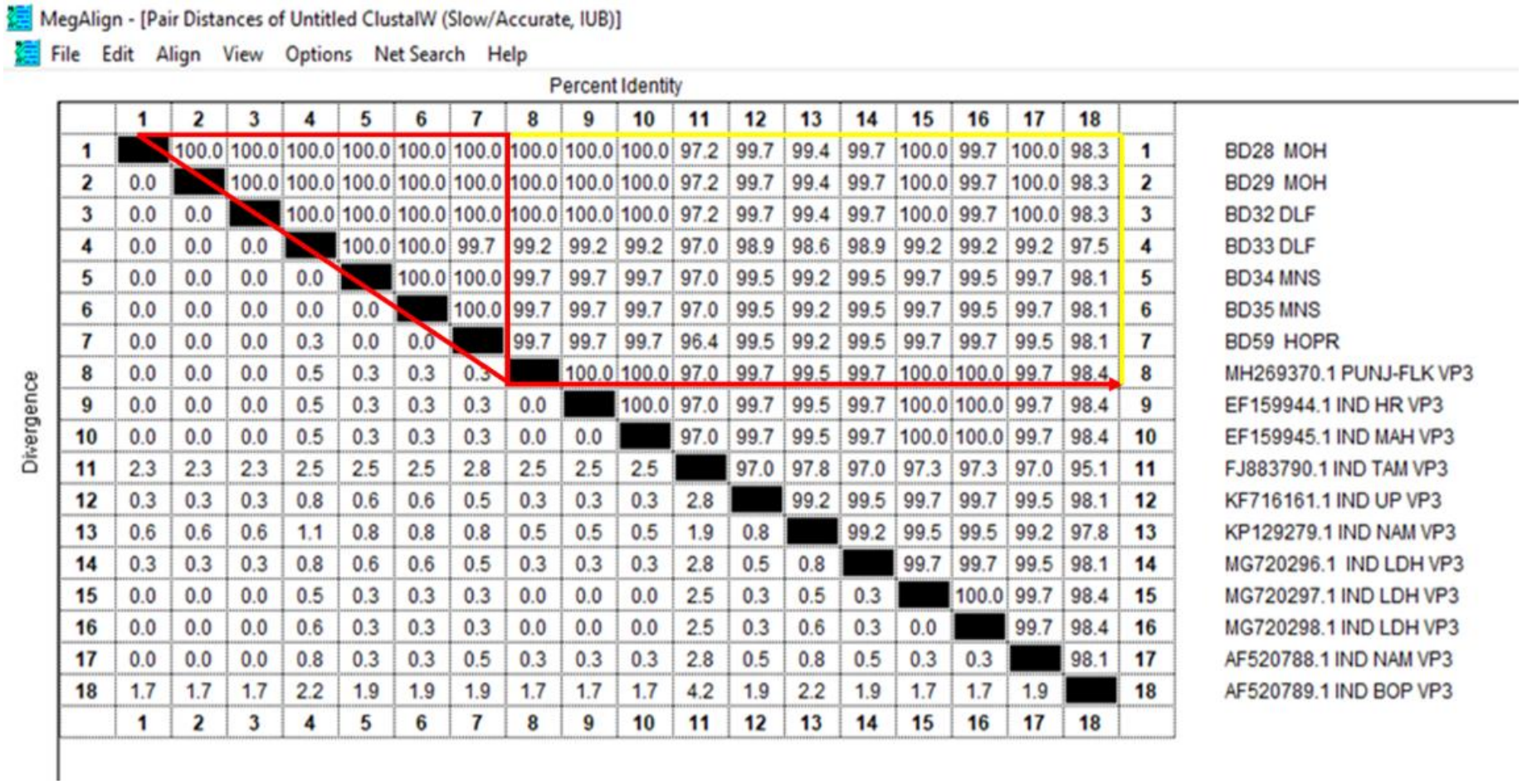


Fig. 16: **Distance matrix of VP2 gene nucleotide sequences of CIAV. Percentage identity/divergence matrix generated by multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of partial VP2 gene nucleic acid sequences. Upper triangle represents the percentage of identity between two viruses. Lower triangle represents the percentage of divergence between two viruses. CIAV isolates from the present study showing 89.9% to 100% identity with reference sequences**



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	99.7	99.4	99.7	100.0	99.7	100.0	98.3	1
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	99.7	99.4	99.7	100.0	99.7	100.0	98.3	2
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	99.7	99.4	99.7	100.0	99.7	100.0	98.3	3
4	0.0	0.0	0.0		100.0	100.0	99.7	99.2	99.2	99.2	97.0	98.9	98.6	98.9	99.2	99.2	99.2	97.5	4
5	0.0	0.0	0.0	0.0		100.0	100.0	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	98.1	5
6	0.0	0.0	0.0	0.0	0.0		100.0	99.7	99.7	99.7	97.0	99.5	99.2	99.5	99.7	99.5	99.7	98.1	6
7	0.0	0.0	0.0	0.3	0.0	0.0		99.7	99.7	99.7	96.4	99.5	99.2	99.5	99.7	99.7	99.5	98.1	7
8	0.0	0.0	0.0	0.5	0.3	0.3	0.3		100.0	100.0	97.0	99.7	99.5	99.7	100.0	100.0	99.7	98.4	8
9	0.0	0.0	0.0	0.5	0.3	0.3	0.3	0.0		100.0	97.0	99.7	99.5	99.7	100.0	100.0	99.7	98.4	9
10	0.0	0.0	0.0	0.5	0.3	0.3	0.3	0.0	0.0		97.0	99.7	99.5	99.7	100.0	100.0	99.7	98.4	10
11	2.3	2.3	2.3	2.5	2.5	2.5	2.8	2.5	2.5	2.5		97.0	97.8	97.0	97.3	97.3	97.0	95.1	11
12	0.3	0.3	0.3	0.8	0.6	0.6	0.5	0.3	0.3	0.3	2.8		99.2	99.5	99.7	99.7	99.5	98.1	12
13	0.6	0.6	0.6	1.1	0.8	0.8	0.8	0.5	0.5	0.5	1.9	0.8		99.2	99.5	99.5	99.2	97.8	13
14	0.3	0.3	0.3	0.8	0.6	0.6	0.5	0.3	0.3	0.3	2.8	0.5	0.8		99.7	99.7	99.5	98.1	14
15	0.0	0.0	0.0	0.5	0.3	0.3	0.3	0.0	0.0	0.0	2.5	0.3	0.5	0.3		100.0	99.7	98.4	15
16	0.0	0.0	0.0	0.6	0.3	0.3	0.3	0.0	0.0	0.0	2.5	0.3	0.6	0.3	0.0		99.7	98.4	16
17	0.0	0.0	0.0	0.8	0.3	0.3	0.5	0.3	0.3	0.3	2.8	0.5	0.8	0.5	0.3	0.3		98.1	17
18	1.7	1.7	1.7	2.2	1.9	1.9	1.9	1.7	1.7	1.7	4.2	1.9	2.2	1.9	1.7	1.7	1.9		18
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

1 BD28 MOH
2 BD29 MOH
3 BD32 DLF
4 BD33 DLF
5 BD34 MNS
6 BD35 MNS
7 BD59 HOPR
8 MH269370.1 PUNJ-FLK VP3
9 EF159944.1 IND HR VP3
10 EF159945.1 IND MAH VP3
11 FJ883790.1 IND TAM VP3
12 KF716161.1 IND UP VP3
13 KP129279.1 IND NAM VP3
14 MG720296.1 IND LDH VP3
15 MG720297.1 IND LDH VP3
16 MG720298.1 IND LDH VP3
17 AF520788.1 IND NAM VP3
18 AF520789.1 IND BOP VP3

Fig. 17: Distance matrix of VP3 gene nucleotide sequences of CIAV. Percentage identity/divergence matrix generated by multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of full length VP3 gene nucleic acid sequences. Upper triangle represents the percentage of identity between two viruses. Lower triangle represents the percentage of divergence between two viruses. CIAV isolates from the present study showing 96.4% to 100% identity with reference sequences. 2.8% divergence with isolate from Tamil Nadu.

Table 28: Detail of references sequence and sequence from the present study used for molecular analysis of VP2

S. No.	ID of Isolates	Accession Number	Geographical location
1.	*GUJ*	FJ498868.1	Gujrat
2.	*TN (2)*	FJ883789.1	Tamil Nadu
3.	*IVRI VP2*	JF712620.1	U.P.
4.	*HR*	EF189155.1	Haryana
5.	*VRDC/CAV/AP*	KJ621017.1	Andhra Pradesh
6.	*VRDC/CAV/HR*	KJ621013.1	Haryana
7.	*TN*	KP129273.1	Tamil Nadu
8.	*VRDC/CAV/VC*	KJ620993.1	VRDC (Pune)
9.	*VRDC/CAV/MH*	KJ620995.1	Maharashtra
10.	*MH*	EF189154.1	Maharashtra
11.	*VRDC/CHD*	MF576068.1	VRDC (Pune)
12.	*LDH*	MG720295.1	Punjab
13.	*ANAND*	EU424059.1	Anand
14.	*PUNJ/FLK*	MH269372.1	Punjab-Fazilka Present study
15.	*VRDC/CHD*	KJ620992.1	VRDC (Pune)
16.	*VRDC/CAV/TN*	KJ620989.1	Tamil Nadu
17.	*ASM*	KX377128.1	Assam

Table 29: Detail of reference sequences and sequences from the present study used for molecular analysis VP3

S. No.	ID of Isolates	Accession Number	Geographical location
30.	*Maharashtra*	EF159945.1	Maharashtra
31.	*BD59*	MK385609	Present study
32.	*PUNJ/FLK*	MH269370.1	Present study
33.	*BD28*	MK385603	Present study
34.	*BD32*	MK385605	Present study
35.	*BD34*	MK385607	Present study
36.	*IND LDH*	MG720298.1	Punjab
37.	*BD35*	MK385608	Present study
38.	*Haryana*	EF159944.1	Punjab
39.	*BD29*	MK385604	Present study
40.	*Namakkal*	AF520788.1	Namakkal
41.	*Bhopal*	AF520789.1	Bhopal
42.	*IND LDH VP3*	MG720296.1	Punjab
43.	*IND LDH*	MG720297.1	Punjab
44.	*BD33*	MK385606	Present study
45.	*UP*	KF716161.1	UP
46.	*INDTN06*	FJ883790.1	Tamil Nadu

4.2.5 Phylogenetic analysis of VP1, VP2 and VP3 gene

Phylogenetic analysis to interpret the evolutionary relationship among the virus isolates was carried out using MEGA7 software and ClustalW algorithm. Maximum likelihood (ML) tree based on retrieved reference nucleotide sequences was constructed using different models.

4.2.5.1 *VP1 gene phylogenetic analysis*

On the basis of complete VP1 gene sequence, two distinct groups were formed by the isolates included in the present study. The isolate from the present study formed distinct cluster along with the reference isolates from Jabalpur, Khasipur, Karnataka, Maharashtra and Punjab. The previously reported isolates from Punjab found closely branched with the isolate from the present study. Isolate from Tamil Nadu and other reference isolates branched distantly from Punjab isolates (Figure 18a). Further analysis with various global isolates showed various distinct cluster formation between the isolates including the isolate from the present study (Figure

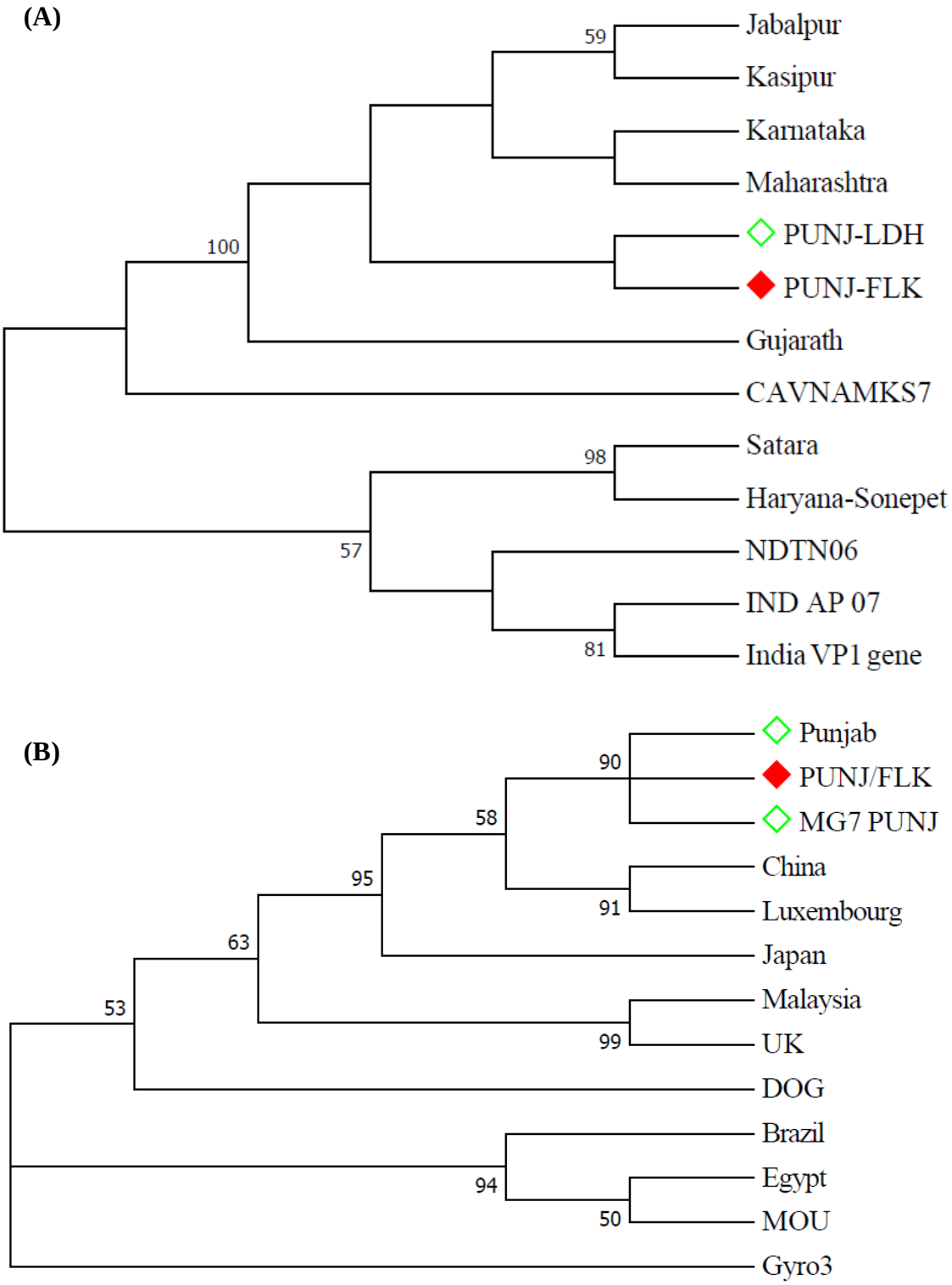


Fig. 18a and 18b: Molecular Phylogenetic analysis of VP1 gene from Indian (A) and Global (B) isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Red box- isolate from present study

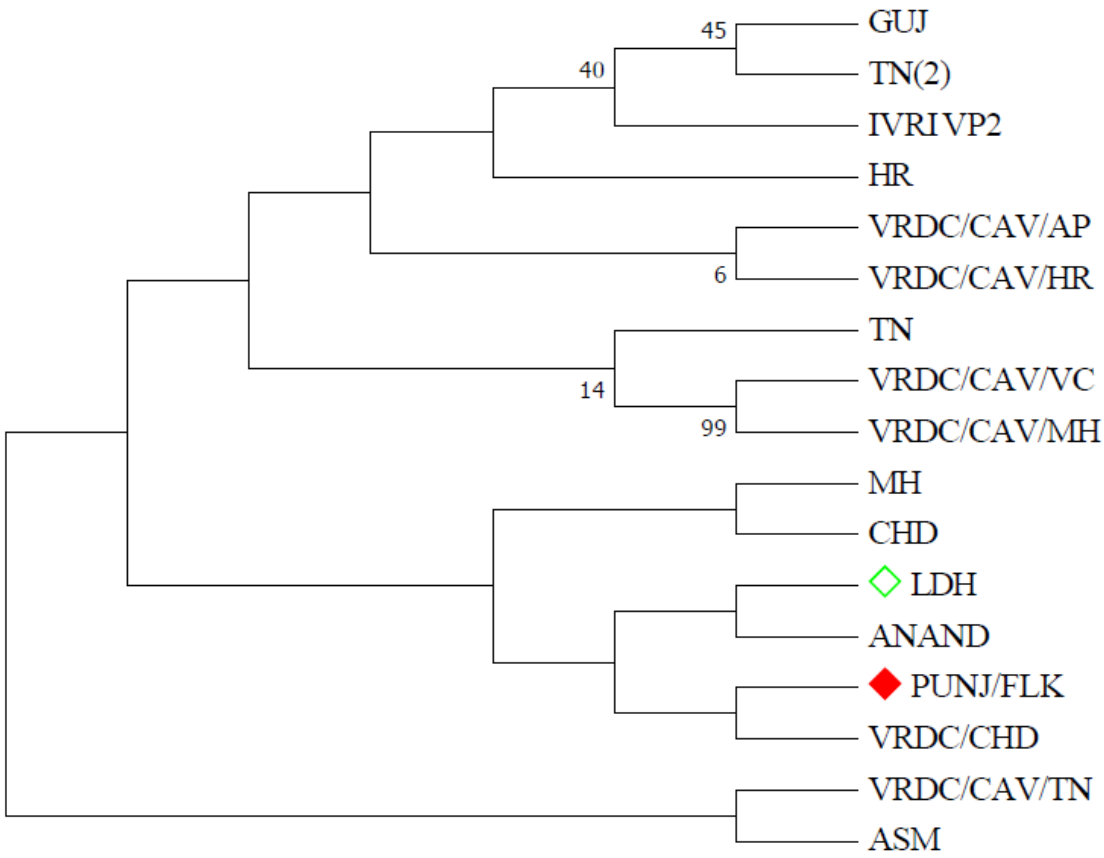


Fig. 19: Molecular Phylogenetic analysis of VP2 gene from Indian isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Red box- isolate from present study.

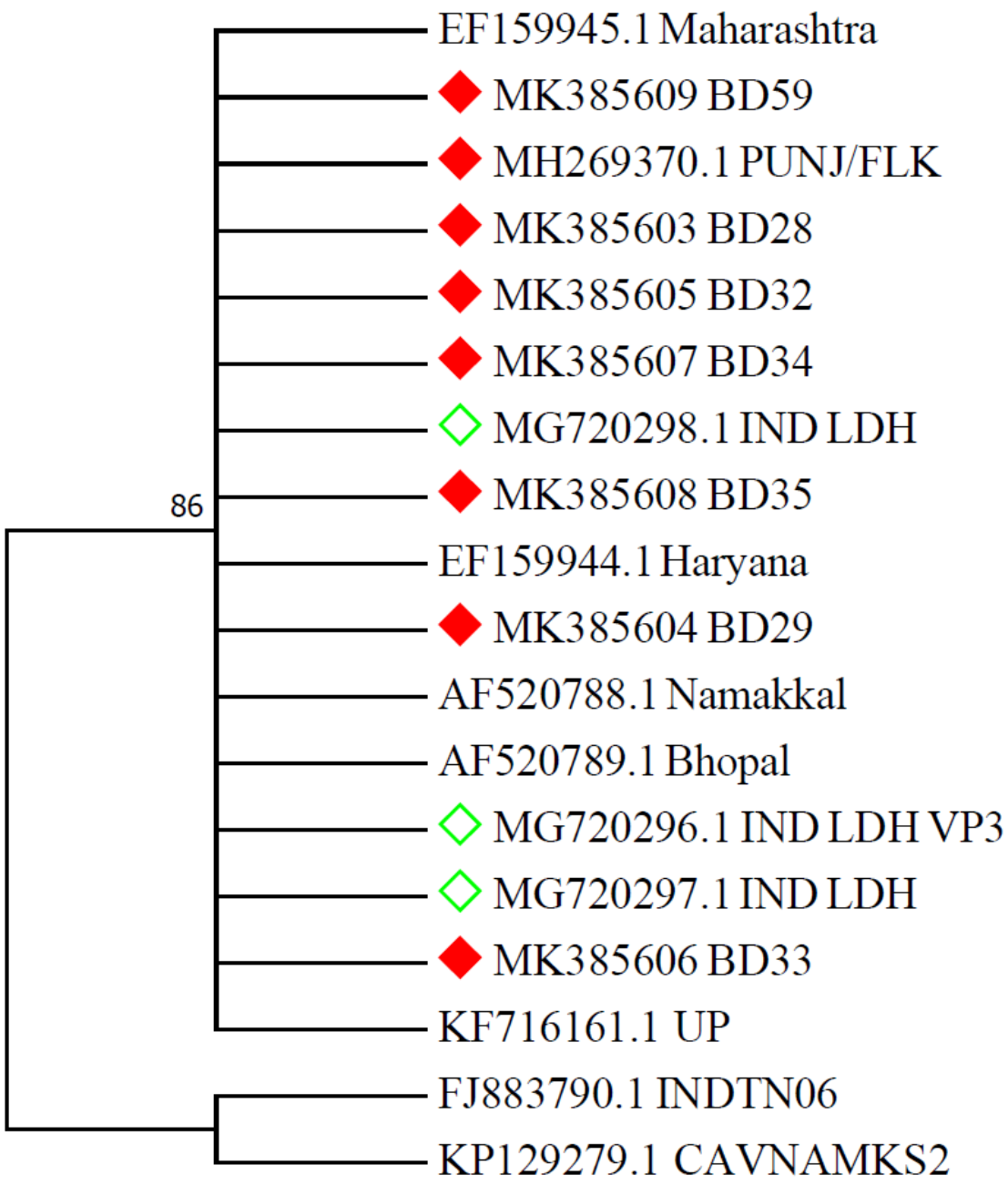


Fig. 20: Molecular Phylogenetic analysis of VP3 gene from Indian isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (values <50 is not shown)

18b). The isolate from the present study along with other Indian isolates formed a single cluster with a bootstrap value 90 which indicates that Indian isolates are closely related but genetically different from other global isolates included in the phylogenetic analysis.

4.2.5.2*VP2 and VP3 gene Phylogenetic analysis*

On the basis of VP2 and VP3 gene sequences, the isolates from the present study did not show distinct Clad/cluster formation along with the reference isolates under the investigation. These isolates also showed close relation to other Indian isolates (Figure 19 and 20).

4.3. Isolation of field strain of CIAV in MDCC MSB1 cell line and its characterization

CIAV was isolated from the field strain circulating among chicken flocks. From the PCR positive samples, 16 highly positive (by DNA concentration) samples were selected for isolation of CIAV. Criteria for selection of sample was based on the concentration of virus load (by DNA concentration) in the samples. A total of 16 positive samples (BD1, BD2, BD3, BD8, BD13, BD14, BD16, BD18, BD19, BD26, BD27, BD39, BD40, BD42, BD43, BD44) were subjected to virus isolation in MDCC MSB1 cell line (Table 30).

Out of 16 field samples processed for virus isolation in cell line, 10 showed characteristic CPE of CIAV infection *viz.* ballooning, enlarged transparent cells, presence of inclusion bodies, blubbing of cell membrane and rupturing of infected cells. The presence of virus was further confirmed by VP1 and VP3 gene specific PCR and demonstration of CIAV antigen by immunofluorescent assay (IFA).

Cell line was sub cultured with an average number of cells ranging from 1.5 to 2 x 10^5 cells/ ml (dye exclusion method – Figure 21) in a 12 well culture plate and 75-80% cell density was observed after 24 to 36hours. Each filtered inoculum (in duplicates) were used to infect the cell line and blindly passaged 7-14 (based on samples) times and observed characteristic cytopathic effects (CPE). Each blind passage took 3-4 days of interval. Characteristic morphological changes of cells were started after 15-20 days (Blind passage No.5) of post inoculation (DPI) in BD1 and BD8 samples and characteristic CPE was observed after 2-3days of passaging. Morphological changes were started by 36-48days (Blind passage No.12) of post infection in BD2, BD3, BD13, BD14, BD16, BD42, BD43, BD44 samples.

Ballooning, enlarged transparent cells, presence of inclusion bodies, blubbing of cell membrane and rupturing of infected cells (Figure 22) were the characteristic CPE observed at blind passage No.14 (42- 56days). Virus isolates showing 80- 90% of CPE were harvested and stored. The samples which were unable to produce any noticeable CPE after blind passage number 14 were considered as negative for viral growth and discarded

Various studies reported the isolation of CIAV from tissues and blood samples (Yuasa, 1983). Initial changes in infected cells were not much appreciable but after passaging for 5-6-time visible damages can be well identified. These changes are suggestive CIAV growth. Cytopathic effect (CPE) produced by the virus can be characterized by observing enlarged, swollen and misshapen cells, cell degeneration and lysis, alkaline red medium, inhibition of cell growth and ultimately the inability to subculture. Similar findings were observed by various other workers also (Noteborn *et al* 1994, Todd *et al* 1999, Renshaw *et al* 1996, Natesan *et al* 2006, Li *et al* 2017).

Noteborn *et al* (1994) showed similar cellular changes in lymphoblastoid cells. After infection, VP3 protein induced apoptin appeared as granules with increased in cell size, and gradually aggregates appeared (prominent at 60hr PI). At this stage of infection, the CIAV-specific cytopathogenic effect was clearly visible.

Li *et al* (2017) isolated CIAV from blood sample and they observed rounding and enlargement of infected cells, lysis or disintegration of cells with reduced number of viable cells. The morphology of uninoculated control MSB1 cells remained normal throughout the study.

The present study also observed similar changes in infected MDCC MSB1 cells. During the initial blind passages, could not recognize growth of virus as MDCC cells overpowered the growth of CIAV. Later the cells started showing enlargement and ballooning (5^{th} passage) after 2-3days that was further passaged for 1-2 more times. As the CIAV were very hardy, as reported earlier, it was found very tedious to make CIAV to adapt and grow in the cell line.

At 7^{th} passage, virus growth in infected cell was appeared as granules formation with increased cell size, and gradually granular aggregates appeared. These findings suggest that characteristic CPE observed can be due to growth of field isolate of CIAV. Further, virus infected cells were subjected to PCR, immunofluorescent staining and electron microscopic observation to confirm the isolated CIAV.

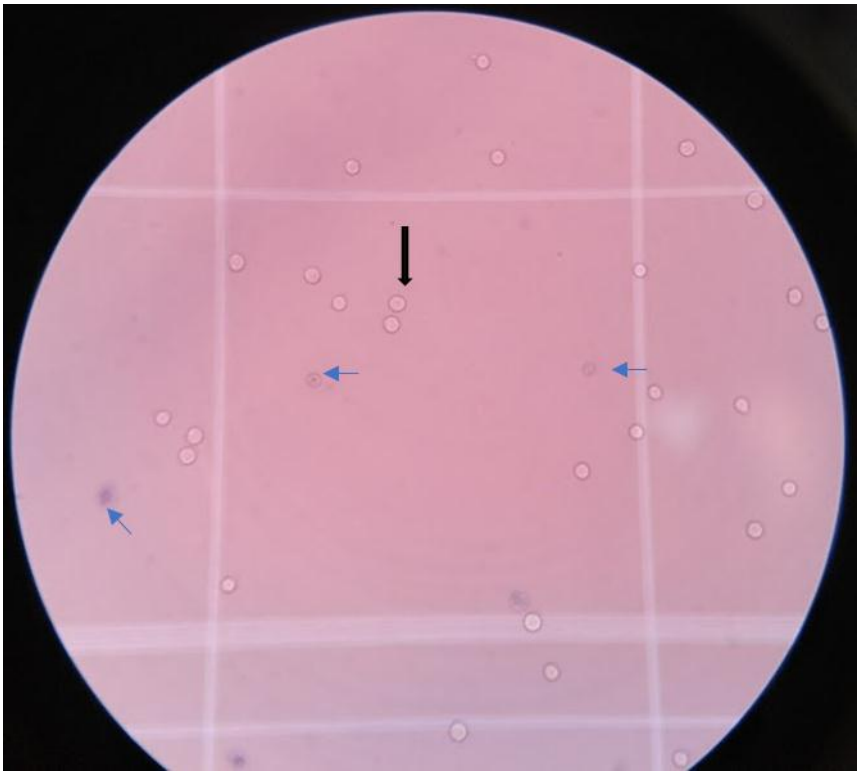


Fig. 21: Trypan blue dye exclusion method for counting of Live cells. Live cells (Black arrow)-Glistening round cells; Dead cells (Blue arrow)- blue stained cells

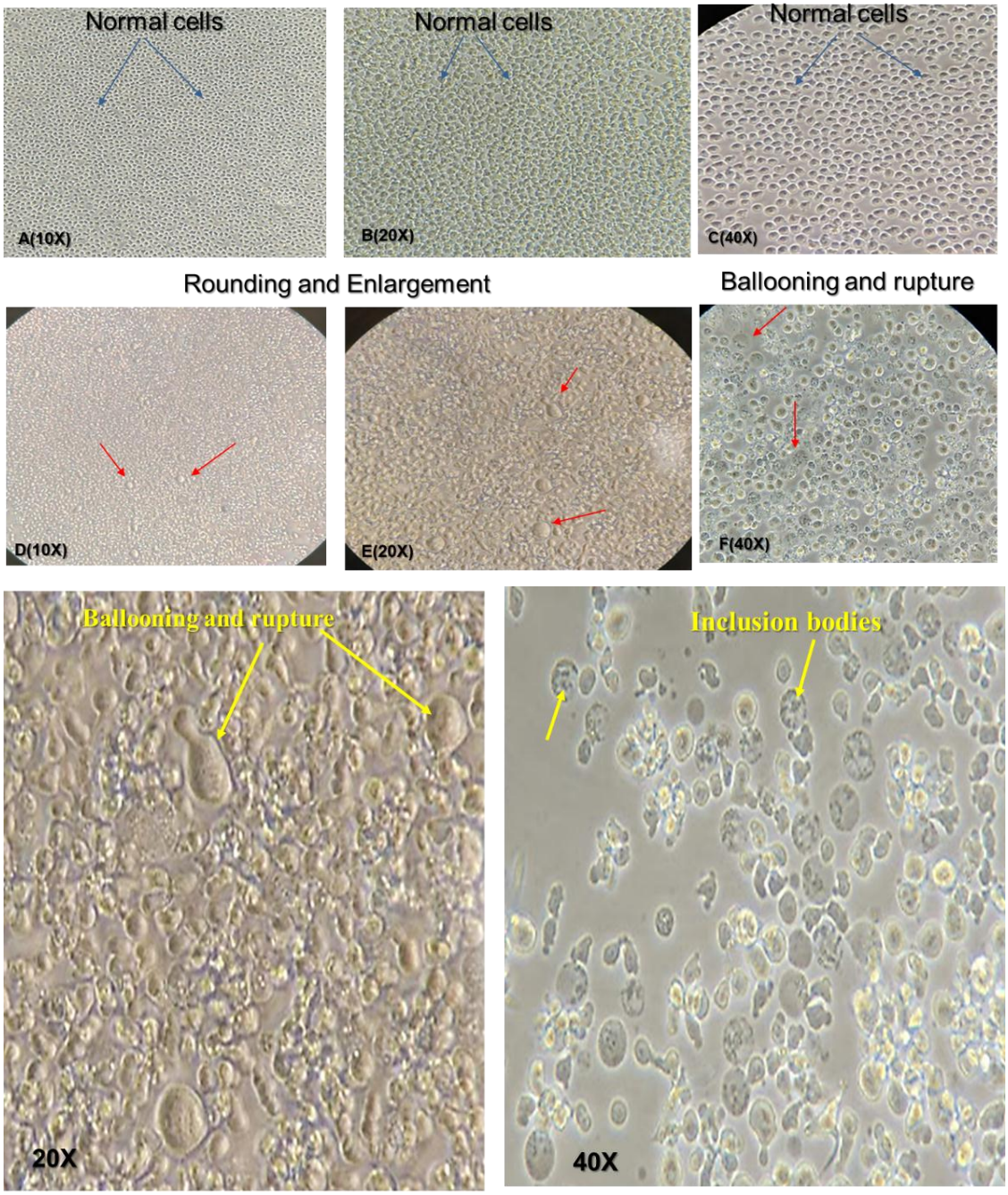


Fig. 22: Cytopathic effects (CPE) produced by CIAV isolates on MDCC MSB1 cells after 24-36 hours post infection. A-C: Normal MDCC MSB1 cells observed with 10X, 20X and 40X objective lenses respectively. D-F: CIAV specific CPE. d)Enlargement of cells (10X), e) Ballooning (20X), f) Aggregation of inclusion bodies Ballooning and rupture (40X). G-H: observation of CPE at higher magnification

Table 30: Blind passage of PCR positive samples in MDCC MSB1 cell line

S. No.	Sample ID	Passage Number												
		P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**	**P13**	**P14**
1.	BD1	N	N	N	N	**Y**	**Y**	**Y**	-	-	-	-	-	-	-
2.	BD2	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
3.	BD3	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
4.	BD8	N	N	N	N	**Y**	**Y**	**Y**	-	-	-	-	-	-	-
5.	BD13	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
6.	BD14	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
7.	BD16	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
8.	BD18	N	N	N	N	N	N	N	N	N	N	N	N	N	N
9.	BD19	N	N	N	N	N	N	N	N	N	N	N	N	N	N
10.	BD26	N	N	N	N	N	N	N	N	N	N	N	N	N	N
11.	BD27	N	N	N	N	N	N	N	N	N	N	N	N	N	N
12.	BD39	N	N	N	N	N	N	N	N	N	N	N	N	N	N
13.	BD40	N	N	N	N	N	N	N	N	N	N	N	N	N	N
14.	BD42	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
15.	BD43	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**
16.	BD44	N	N	N	N	N	N	N	N	N	N	N	**Y**	**Y**	**Y**

'N' =No cytopathic effect observed, 'Y' = cytopathic effect observed , '-' = No further passaging done

4.3. 1 Confirmation of cell culture isolates by VP1 and VP2 gene specific PCR

The 10 virus isolates which showed characteristic CPE in MDCC MSB1 cell line were subjected to VP1 and VP3 gene specific PCR. As VP3 gene is the highly conserved gene in CIAV genome, initial PCR amplification was done with VP3 specific primer. After that VP1 gene specific primer was used to amplify the CIAV DNA for further confirmation. PCR products of both VP1 and VP3 gene were visualized by agar gel electrophoresis. It gave expected amplicon size of 1350bases and 364bases of VP1 and VP3 genes respectively (Figure 23a and 23b).

Various studies reported the isolation of CIAV in cell culture like MDCC MSB1 and characterized viral genome. CIAV is unique for its amino acid composition which found to be remarkably conserved even in isolates from different parts of the world. Renshaw *et al* (1996) have characterized field isolates of CIAV which vary significantly in terms of their abilities to replicate in cell culture, demonstrating a biological difference between isolates.

4.3.2 Confirmation of viral antigen by Immuno Fluorescent assay (IFA)

The presence of viral antigen in the infected MDCC-MSB1 cells was confirmed by indirect immunofluorescent staining. The characteristic intra cellular greenish yellow fluorescence of antigen was observed in all 10 isolates of CIAV. There was no fluorescence observed in control cells. Various dilutions of anti-CIAV antibody and FITC conjugate was used to stain the virus infected cells after fixing with cold methanol and acetone. The characteristic fluorescence of FITC was clearly visible in the enlarged granulated cells (Figure 24).

Dhama (2002) and Wani *et al* (2014) in separate study demonstrated virus-specific antigen in infected MSB1 cells and cryostat-sections of infected tissues by indirect immunofluorescence staining. Fluorescent staining of small, irregularly shaped granules in the nucleus of enlarged cells is observed in positive immunofluorescence demonstrating viral antigens. Employing monoclonal or polyclonal antibodies to apoptin protein produced immunofluorescence which can be characterized by doughnut shaped granular apoptic bodies. In the present study also observed similar findings which confirm the presence of viral antigen in the cell culture isolates

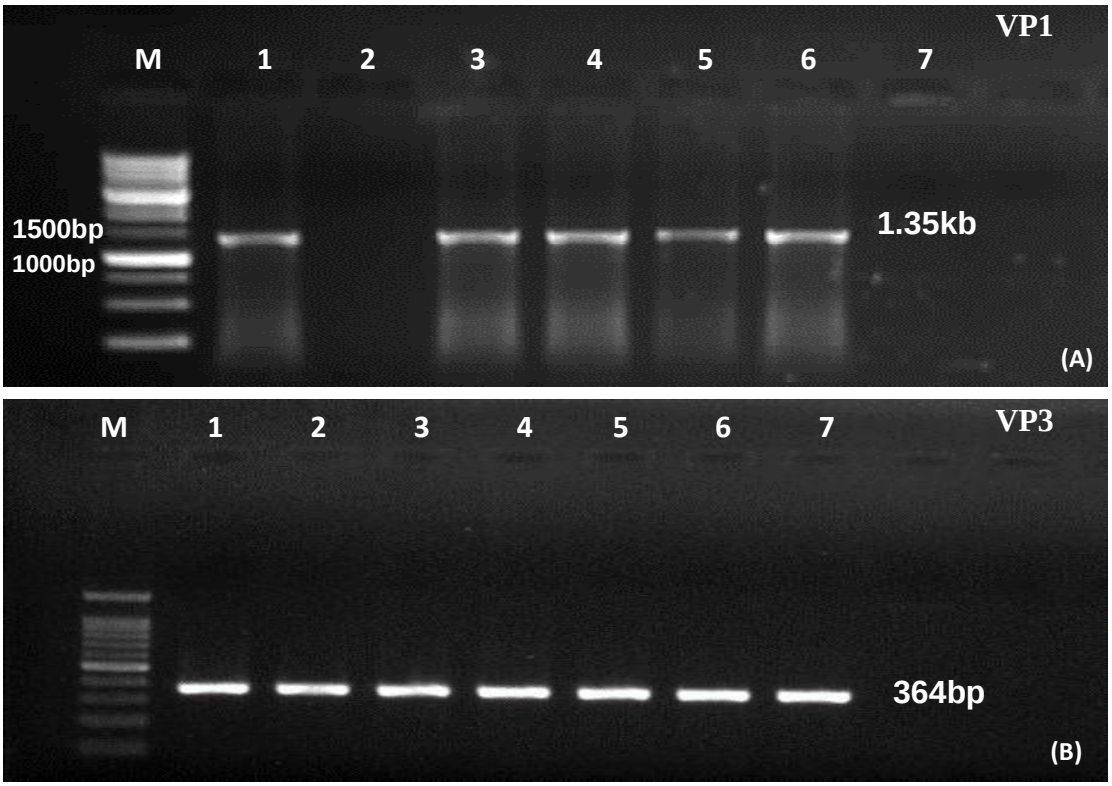


Fig. 23a and 23b: Agarose gel electrophoresis of PCR amplifying VP3 and VP1 gene of CIAV cell culture isolates. a) VP3 gene specific DNA (364bsases), b) VP1 gene specific DNA (1350bases)

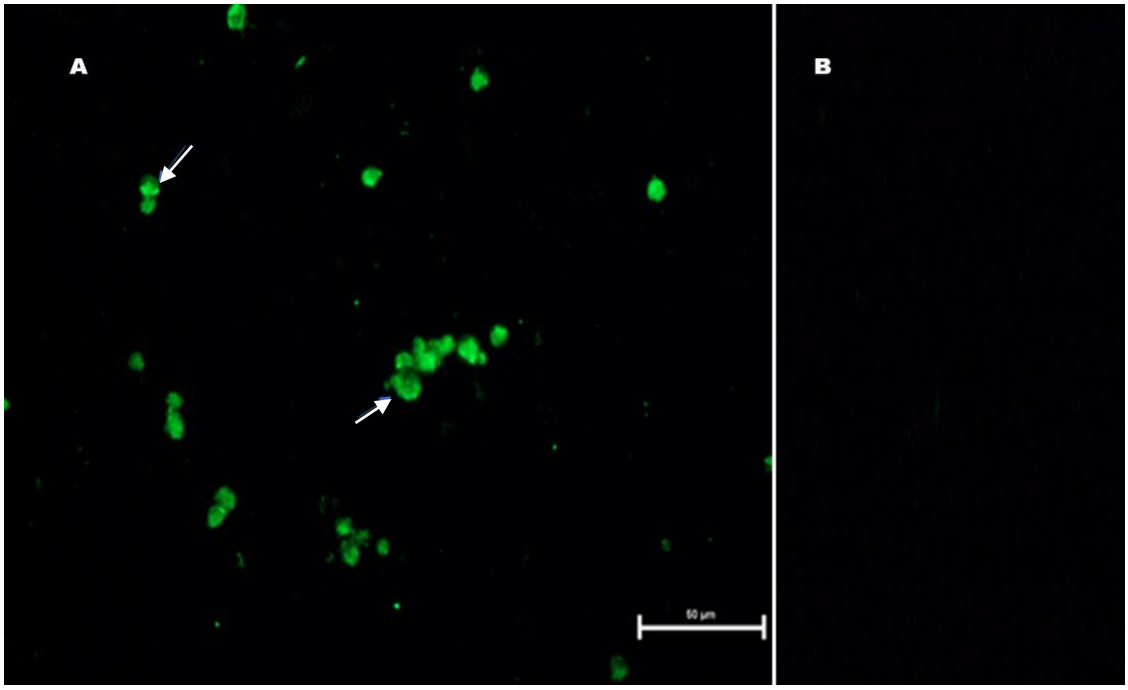


Fig. 24: Confirmation of CIAV antigen by immunofluorescent assay(IIFA).

A) Fluorescence of FITC in the granulated cells. B) Negative control

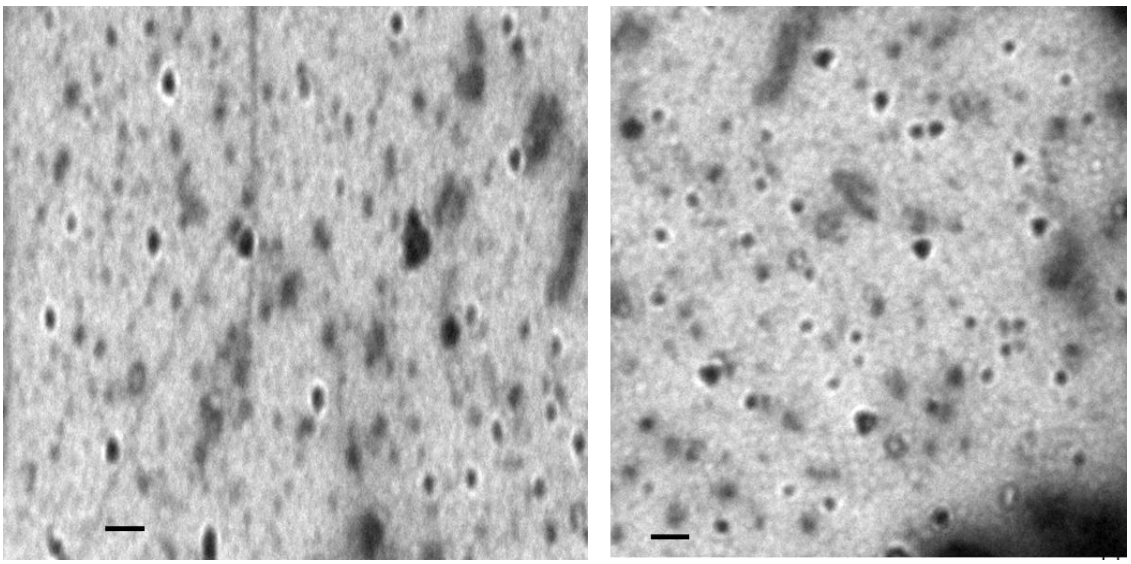


Fig. 25: Confirmation of CIAV isolates by Electron microscopy. Electron dense small round particles approximately 25nm size (Bar -50nm).

4.3.3 Confirmation of CIAV particles by Electron microscopy

Negatively stained virus suspension showed various electron dense particle with size varying from 25 to 26nm approximately in diameter (Figure 25). The individual surface morphology was not clear in electron microscopic image obtained in the present study. Based on CPE in cell culture, genome demonstration by PCR, sequencing and approximate size observed in electron microscopy, the electron dense particles observed were indicative of CIAV. Further studies need to be carried out with purified virus particle to obtain a clear surface topography of CIAV isolated in cell culture.

Various electron microscopic analysis has been done earlier to demonstrate the surface topography and morphology of CIAV (Goryo *et al* 1987, McNulty *et al* 1990, Noteborn *et al* 1994, Crowther *et al* 2003). The size of the CIAV particle reported were ranged from 19 nm to 26nm approximately. Our finding also agrees with the similar results but could not get clear morphological structure of the virus.

4.4 Whole genome amplification of CIAV DNA

The whole genome of CIAV comprises of 2.3kb circular ssDNA. PCR amplifying the entire length of CIAV genome was optimized and genome was further characterized by cloning and sequencing. For amplifying the whole genome of CIAV, a temperature gradient ranging from 50 - 60°C was used to optimize the annealing temperature (Ta). An amplicon size approximately 2.3kb was observed at temperature range between 52-56 °C after resolving in agar gel electrophoresis. In the present study, a temperature of 54°C was selected (as annealing temperature) for further amplification of full length CIAV genome. Further, whole genome of cell culture isolates was amplified using DNA extracted from the cell culture isolates (n = 10) and all the 10 isolated viruses gave CIAV genome specific band after resolving in agar gel electrophoresis (Figure 26).

4.4.1 Cloning and sequence analysis

The purified PCR amplicon were cloned into pGEM®-T Easy vector (Figure 27). The clones were screened for target insert ligated to the vector by blue-white screening (Figure 28) and colony PCR using whole genome specific primers (Figure 29). Out of 10 cell culture isolates of CIAV, only 3 isolates (BD1, BD2 and BD44) produced the characteristic white colonies in LB agar plates. The positive and back

ground control plates were also included which also produced blue white and blue colonies respectively. The white colonies in the test plates were screened by colony PCR which given expected amplicon size of 2-2.3Kb in agarose gel electrophoresis. After confirmation, plasmid was extracted from the overnight grown bacterial culture and sequencing was done in both direction by commercial out sourcing.

4.4.2 Molecular characterization of partial/ whole genome of CIAV

4.4.2.1 Sequence analysis of CIAV genome

Molecular detection and characterization of the CIAV has been carried out by PCR and sequencing of CIAV genome. CIAV genome is highly conserved throughout the length of the DNA except VP1 gene coding region which have maximum nucleotide variation. Among the three genes of CIAV, hypervariable region is residing in the VP1 gene and it transcribe into highly immunogenic protein (Todd *et al* 1990, Renshaw *et al* 1996, Li *et al* 2017) that form the base of capsid in matured virus particle. The immunogenicity and virulence of the virus is determined by the presence few amino acid residues in the VP1 gene coding region. Therefore, monitoring of CIAV circulating in the field at various interval of time is necessary to determine the genotype and pathotype present in the field.

In the present study cloned plasmid containing CIAV genome were sent for sequencing by commercial out sourcing. Sequence of CIAV BD1 isolate obtained in the present study was named as BD1/PUNJ/FSLK. The sequence was first analyzed by BLAST (NCBI) analysis which showed high percentage of similarity with other sequences of CIAV isolates available in the GenBank. After sequencing, we obtained a partial sequence of BD1/PUNJ/FSLK which is composed of 1.753Kb of CIAV. Further sequence was analyzed by DNASTAR and MEGA.7 software to infer the homology and phylogenetic relationship with published genome sequences of CIAV, retrieved from GenBank (NCBI) (Table 31).

The sequence consists of three overlapping ORF of VP1 VP2 and VP3 gene. VP2 and VP3 genes consist of complete coding sequence whereas VP1 gene was incomplete at 3’ end. There were many point mutations/substitution that could be observed in the various region of the genome sequence obtained (Table 30) which consequently led to amino acid changes in the coding region like Isoleucine to Leucine (I to L), Serine to Glycine (S to G) and Alanine to Serine (A to S) (Figure 30).

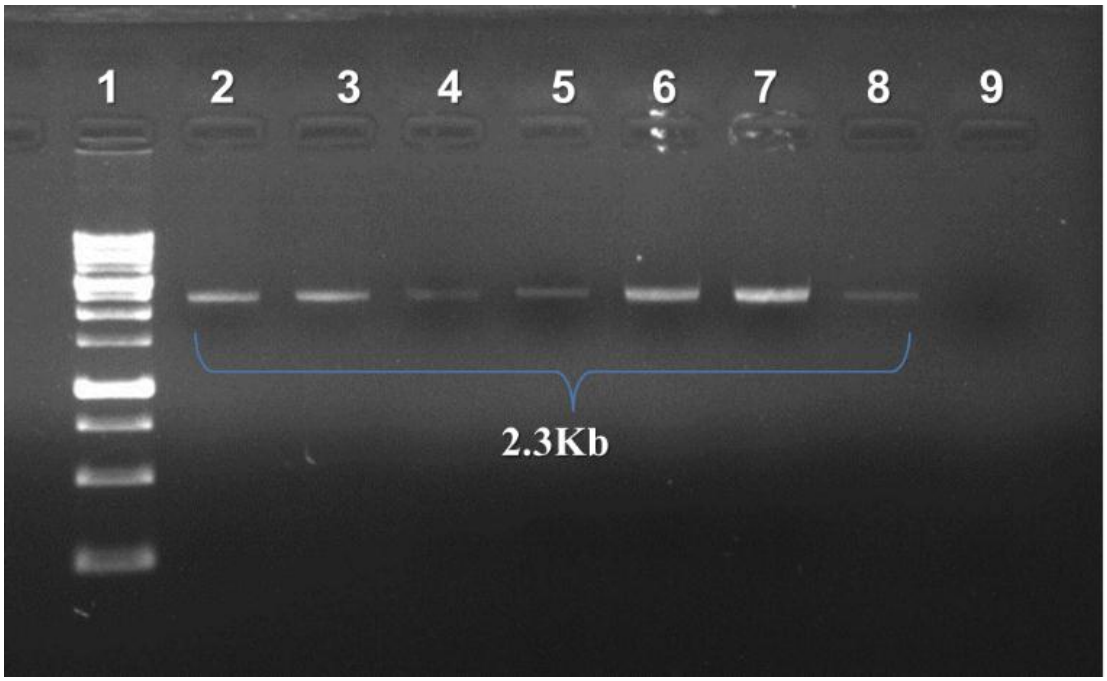
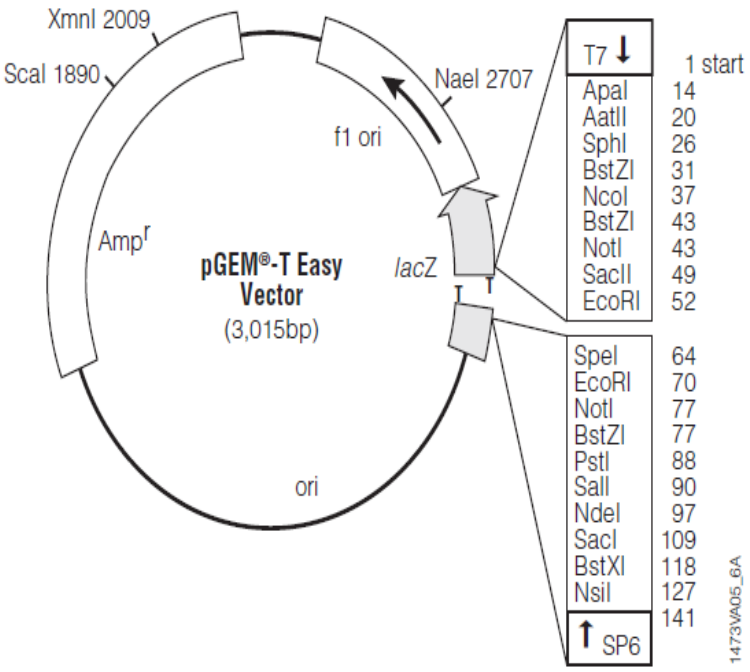


Fig. 26: Agarose gel electrophoresis of PCR amplifying whole genome of CIAV cell culture isolates.

Lane 1:Marker (1kbp); Lane 2: Positive control; Lane 3-8 : cell culture isolates, Lane 9= NTC



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Fig. 27: Map of pGEMT easy vector.

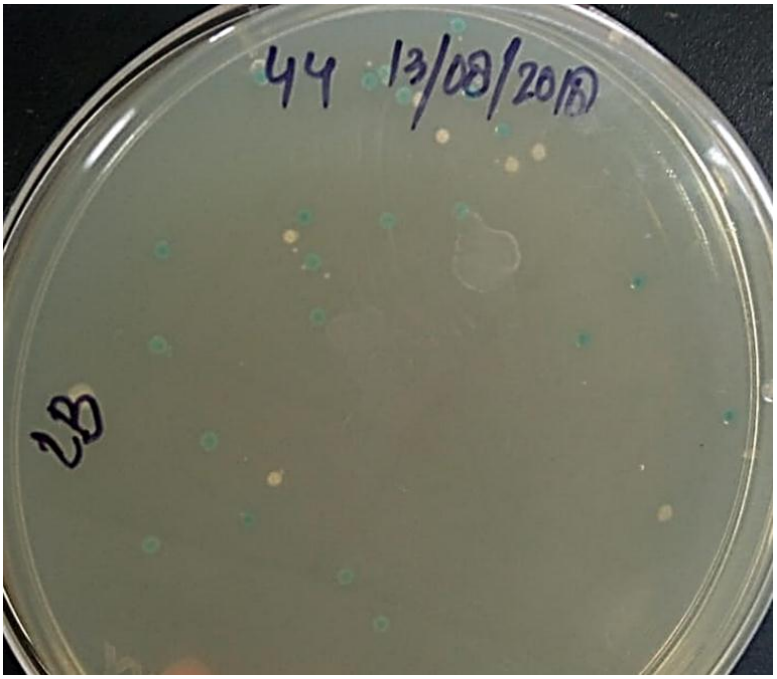


Fig. 28: Blue white screening of bacterial colonies with target insert.

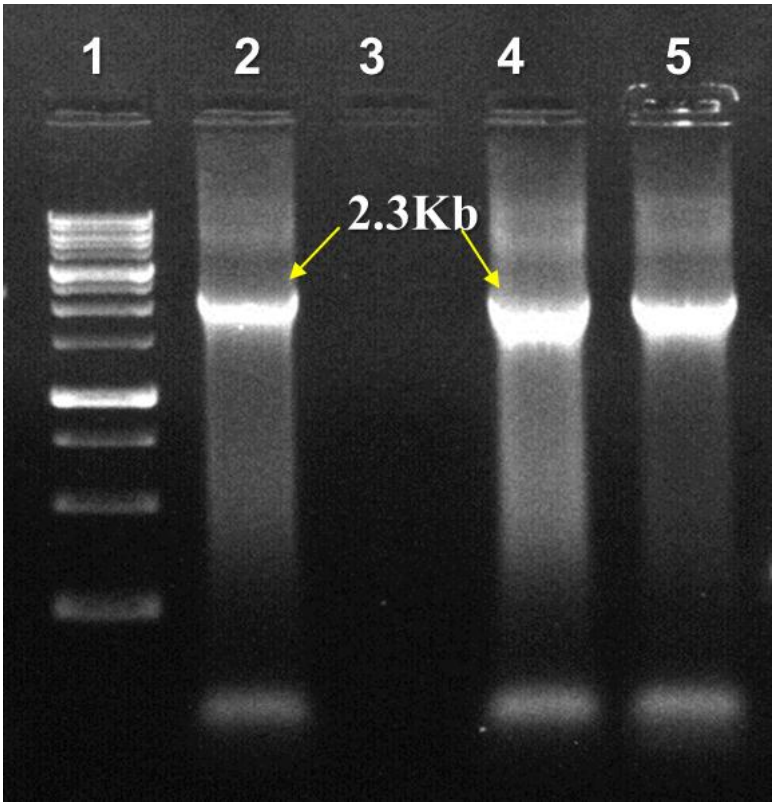


Fig. 29: Agarose gel electrophoresis of Colony PCR. Amplicon specific for whole genome 2.3Kb of CIAV

Lane 1: Marker (1kbp); Lane 2,4 and 5: colony PCR (BD1, BD2 and BD44); Lane 3: NTC

Table 31: Different nucleotide changes observed in genome of CIAV

S. No.	Nucleotide substitution or mutations	Nucleotide position (consensus position)
1.	A to G	804(1162)
2.	G to A	829(1189), 1602(1960), 1761(2119)
3.	A to C	847(1205), 1587(1945),1677(2035)
4.	T to C	909(1267),1257(1615),1305(1663)1449 (1807), 1608(1966),1617(1975), 1710(2068)
5.	C to A	1326(1684), 1600(1958)
6.	A to T	1437(1795)
7.	C to T	1494(1852), 1590(1948),
8.	G to A	1711(2069)

Based on the full-length gene sequence information retrieved from GenBank, the complete nucleotide sequences of VP1, VP2 and VP3 genes were determined. The coding region of VP1 gene was incomplete with few numbers of nucleotides at 3' end whereas VP2 and VP3 genes had full length nucleotides consisting of 652 and 364 long, respectively. No insertion or deletion of nucleotides were observed in this region. Nucleotide sequence alignment of different coding region of sequence showed various nucleotide mutation and substitutions in VP1 gene (Table 32).

Table 32: Nucleotide changes in VP1 coding region of BD1/PUNJ/FSLK isolate

S. No.	Nucleotide substitution or mutations	Nucleotide position
1.	A to C	373,1113,1203
2.	T to C	783, 831
3.	C to G	860
4.	A to T	963
5.	C to T	1116
6.	C to A	1126
7.	G to T	1237

The mutations were observed at 783, 831, 963,1116 and 1126 nucleotide positions of VP1 gene coding region. Deduced Amino acid alignment of VP1 gene coding regions of CIAV genome showed various amino acid substitution at 125(I to L), 139(Q to K), 144(Q to E), 288(A to S), 377(L to I), 394 (Q)and 414 (A to S). The hypervariable region of VP1 gene that range from position 139 to 151 showed two amino acid substitution at 139 and 144 position. Major genetic determinant of virulence is based on the residue at 394 in VP1gene. A glutamine (Q) residue at this position indicate high virulence and histidine (H)for low virulence. The isolate from the present study comprises a glutamine at position394, suggesting that it represent highly pathogenic virus. Also, the growth and spread of virus is determined by the glutamine at 139 and/or at 144 which consider that these residues are associated with decreased spread of the virus (Renshaw *et al* 1996, Zhang*et al* 2013, Li *et al* 2017). Also, few reports suggest that nucleotide at 75I, 89T, 125L, 141L, and 144E associated with lower pathogenicity (Todd *et al* 2002, Kye *et al* 2013). In the present study the nucleotides at these positions observed were 75V, 89T, 125L, 141Q and 144E.There was no Nucleotide substitution or mutations were observed in VP2 and VP3 coding region of CIAV genome of BD1/PUNJ/FSLK isolate.

Various research works have been done to characterize the genome of CIAV from different part of the world (Todd *et al* 1995, Renshaw *et al* 1996, Yamaguchi *et al* 2001, Simionatto *et al* 2006 Zhang *et al*2013, Li *et al* 2017) which showed that biological variation and genetic difference exists among various isolates.

Renshaw *et al* (1996) showed that MDCC-MSB1 cell lines vary in their susceptibilities to infection with different CIAV isolates and that can affect both the ability to grow and the rate of replication in different sublines. The association ofVP1 amino acid residues at Q-139 and/or Q-144 with a decreased rate of spread was revealed from the sequence analysis. But CIAV isolates show little divergence in most coding regions. Even though, there was considerable difference at nucleotide positions (42) in ORF1, it results only four amino acid differences in the predicted protein. Also, they suggest that mutation can occur in CIAV during various passage in cell culture.

Yamaguchi *et al* (2001) studied pathogenicity of genetically modified DNA clone of CIAV. The point mutation at amino acid residue 394 is crucial in eliciting pathogenicity of the virus. The cloned viruses with glutamine at this position were highly pathogenic, whereas those with histidine had low pathogenicity.

Zhang *et al* (2013) discovered 92 variable residues in the CIAV genome and reported evidence of intra-group genetic recombination which plays a role in generating genetic diversity in natural populations of CIAV. All the isolates had glutamine at 394 position- a genetic determinant of high pathogenicity.

Li *et al* (2017) studied recombination events in CIAV isolates including mouse and dog. Along with previous reports (Eltahir *et al* 2011a, Eltahir *et al* 2011b, Zhang *et al* 2013), the results may highlight the important role of genetic recombination events in shaping the genetic diversity of CIAV.

Multiple sequence alignment of BD1/PUNJ/FSLK sequence with reference sequences revealed that highest percentage identity with sequence from South Korea (98.9%) and Taiwan (98.8%). BD1/PUNJ/FSLK showed 98.2% identity with Indian isolate from Tamil Nadu (Figure 30). (Sequence analysis-Annexure II and II)

4.4.2.2 Phylogenetic analysis

Phylogenetic analysis to deduce the evolutionary relationship among the virus isolate was carried out by using MEGA7 software and ClustalW algorithm. Maximum likelihood (ML) tree was constructed based on reference nucleotide sequences using Tamura-Nei model (Table 33). On the basis of partial genome sequence generated in the present study, the isolates were segregated into three distinct cluster and BD1/PUNJ/FASLK closely branched with isolates from south Korea and Taiwan (Figure 31). Other isolates one from India (Tamil Nadu), Malaysia, China were found in two other clusters. USA isolate was found distantly related to BD1/PUNJ/FASLK.

The genome of CIAV has been analysed by various researchers. Isolates from the various region of the world show limited sequence variability. All isolates, perhaps, belongs to single serotype but different genotypes. The whole genome analysis of various isolates shows that there could be intergenotypic recombination event that may decide the virulence of virus. Kim *et al* (2010) from South Korea have analysed hypervariable region of the CIAV genome.

Table 33: Detail of reference sequences and sequence from the present study used for molecular analysis of partial sequence of CIAV isolate

S. No.	Isolate ID	Accession Number	Geographical area
1.	*BD1/PUNJ/FSLK*		Punjab-Present study
2.	*south korea wg*	*JF507715.1*	South Korea
3.	*Taiwan WG*	KJ728830.1	Taiwan
4.	*DQ401malaysia WG*	DQ217401.1	Malaysia
5.	*AF908china WG*	AF475908	China
6.	*WG TN*	KY053900	Tamil Nadu
7.	*cux1 WG*	M55918.1	USA
8.	*AF038 malaysia WG*	AF390038	Malaysia
9.	*JQ762china WG*	JQ762	China
10.	*DQ400 malayisia WG*	DQ217400.1	Malaysia
11.	*AF900 USA WG*	AF311900	USA

The results of both nucleotide and amino acid sequence comparison indicated that Korean strains were separated into different groups. AboElkhair *et al* (2014) did analysis of nucleotide sequence and deduced amino acid showed that CIAV detected was not related to vaccine strain but can be a field strain. The phylogenetic analysis from the deduced amino acid showed grouping of virus in to one group with other reference isolates. Olszewska-Tomczyk *et al* (2016) from Poland studied Phylogenetic analysis of 16 CIAV isolates circulating in the country. Sequence analysis of CIAV isolates with various globally reported sequences revealed a low genetic variation at the nucleotide level. Ou *et al* (2018) characterised field isolates of CIAV circulating in Taiwan Phylogenetic analysis of the VP1 gene revealed that Taiwanese isolates belonged to different genotypes. Taiwanese isolates showed close relation with vaccine strains (26P4 or Del-Ros) used in the country. They suggest that inter-genotypic recombination may be occurred between viruses of different genotypes. Therefore, sequence analysis and phylogenetic analysis of the isolates from the present study clearly indicate that CIAV is circulating among chicken flocks of Punjab state and isolates are closely related to other reported CIAV isolates from various parts of the India and the world.

MegAlign - [Pair Distances of aligned with NT position 1 to 2.3kb after deletion of other few seq.meg ClustalW (Slow/Accurate, IUB)]

File Edit Align View Options Net Search Help

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	
1		96.6	96.9	96.7	98.3	96.6	96.6	96.5	97.1	96.4	96.3	1
2	3.4		98.3	98.1	96.3	98.0	98.0	97.7	99.2	97.6	97.9	2
3	3.2	1.8		98.3	96.7	98.1	98.3	98.2	98.7	97.9	98.3	3
4	3.3	1.9	1.7		96.4	99.2	98.4	98.2	98.5	97.7	98.0	4
5	1.6	3.8	3.3	3.7		96.3	96.3	96.1	96.8	96.1	96.2	5
6	3.4	2.0	1.9	0.8	3.8		98.3	98.0	98.5	97.5	98.1	6
7	3.4	2.1	1.7	1.6	3.8	1.7		99.0	98.4	98.2	98.9	7
8	3.5	2.3	1.8	1.8	3.9	1.9	1.0		98.1	97.8	98.8	8
9	2.9	0.8	1.3	1.5	3.3	1.6	1.6	1.9		98.0	98.2	9
10	3.7	2.4	2.1	2.3	4.0	2.5	1.9	2.2	2.0		97.8	10
11	3.8	2.2	1.8	2.1	3.9	1.9	1.1	1.3	1.8	2.2		11
	1	2	3	4	5	6	7	8	9	10	11	

1 AF311900.3 USA WG
2 AF390038.1 malaysia WG
3 AF475908.1 china WG
4 AY040632.1 complete genome
5 DQ217400.1 malayisia WG
6 DQ217401.1 malasysia WG
7 JF507715.1 south korea wg
8 KJ728830.1 Taiwan WG
9 KY053900.1 WG TN
10 KY486155.1 complete genome
11 BD1 PUNJ FSLK

Fig. 30: Distance matrix of nucleotide sequence of CIAV genome. Percentage identity/divergence matrix generated by multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of CIAV partial nucleic acid sequences (BD1/PUNJ/FSLK). Upper triangle represents the percentage of identity between two viruses. Lower triangle represents the percentage of divergence between two viruses. CIAV isolate from the present study showing 98% identity with South Korea and Taiwan isolates.

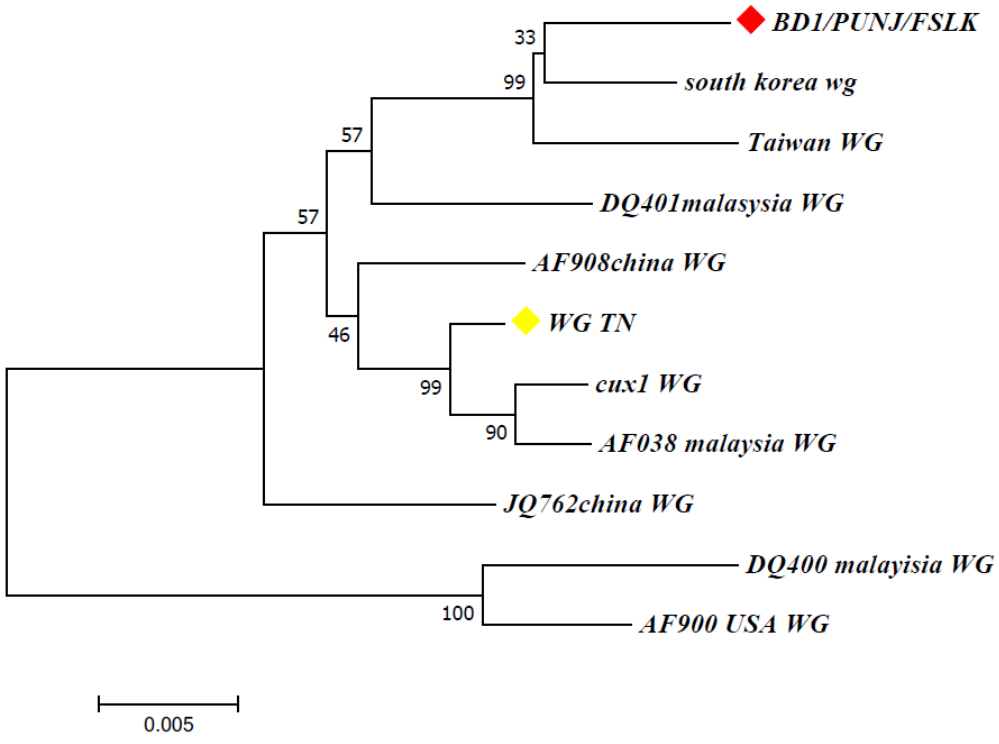


Fig. 31: Molecular Phylogenetic analysis of CIAV genome. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. BD1/PUNJ/FSLK grouped with south korea and Taiwan with bootstrap value of 99

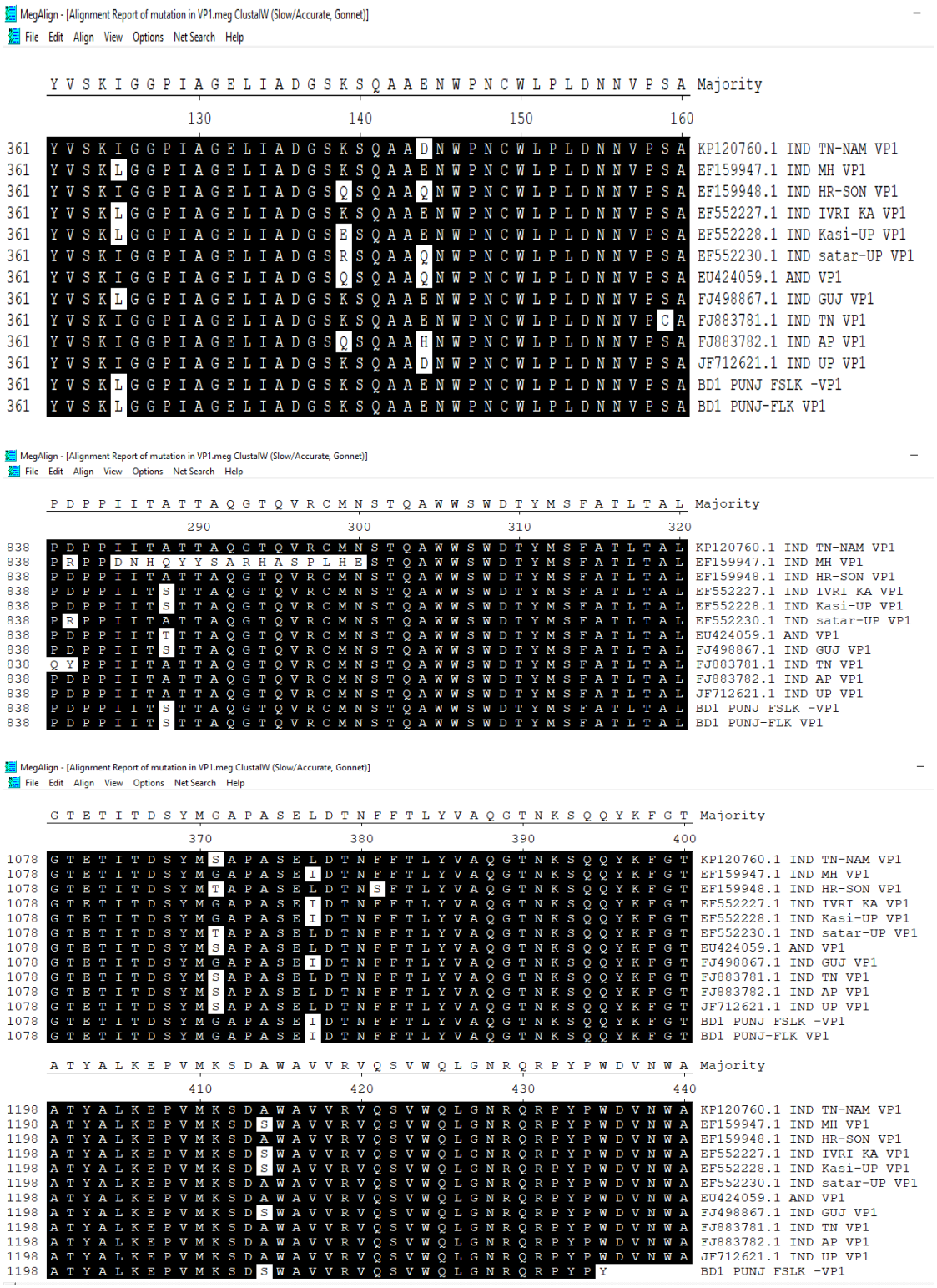


Fig. 32: Amino acid alignment of coding region of VP1 protein. Multiple alignments using ClustalW algorithm (MegAlign, DNASTAR) of partial VP1 gene amino acid sequences deduced from genome of BD1/PUNJ/FSLK isolate. K-139, E-144, S-288, I-387, Q-394 and S-414

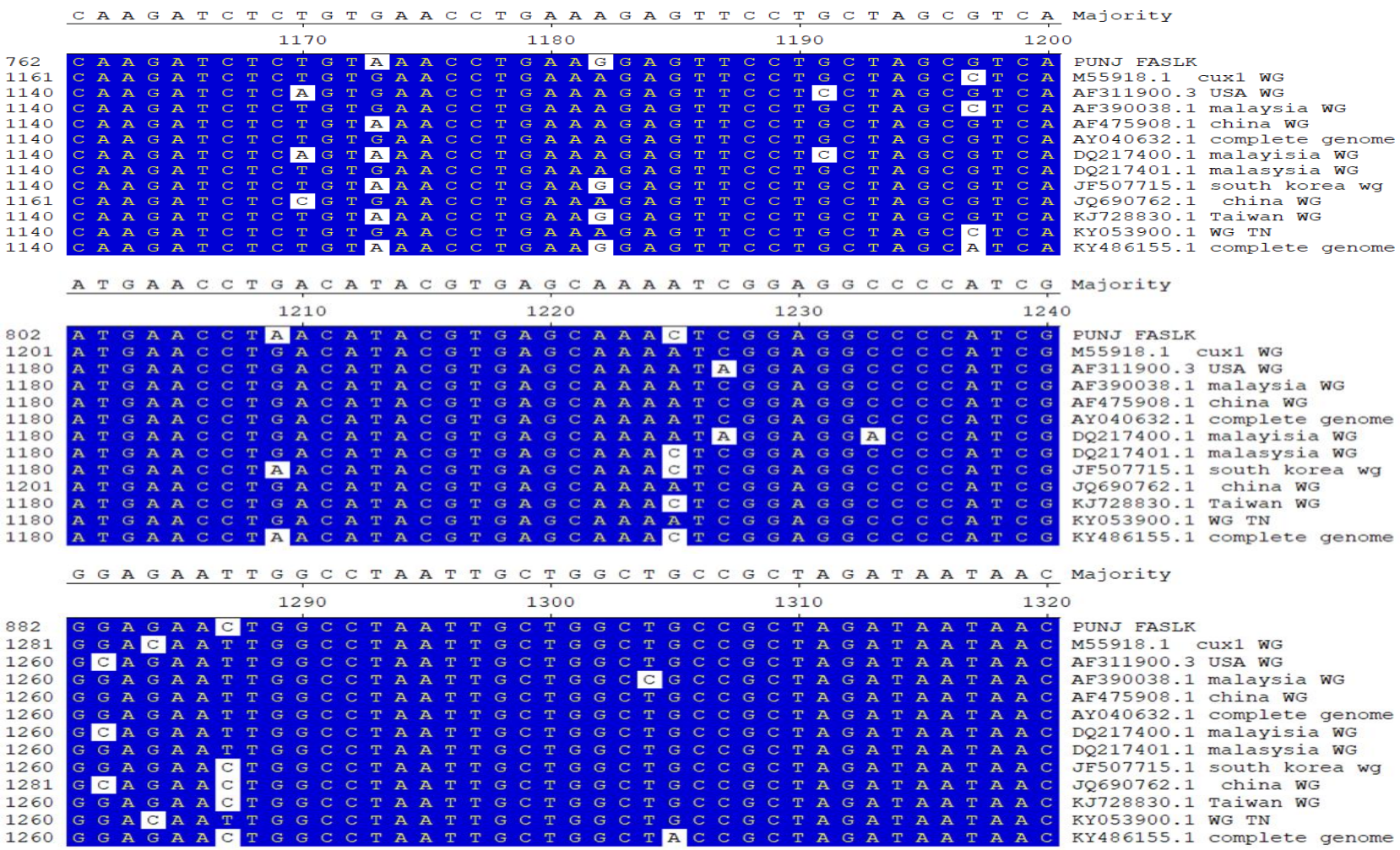


Fig. 33: Nucleotide sequence alignment of BD1/PUNJ/FSLK with reference sequences. Multiple alignments using ClustalW algorithm (MegAlign, DNASTAR). Various substitution/point mutation of BD1/PUNJ/FSLK isolate.

contd…

Fig. 33 Contd..

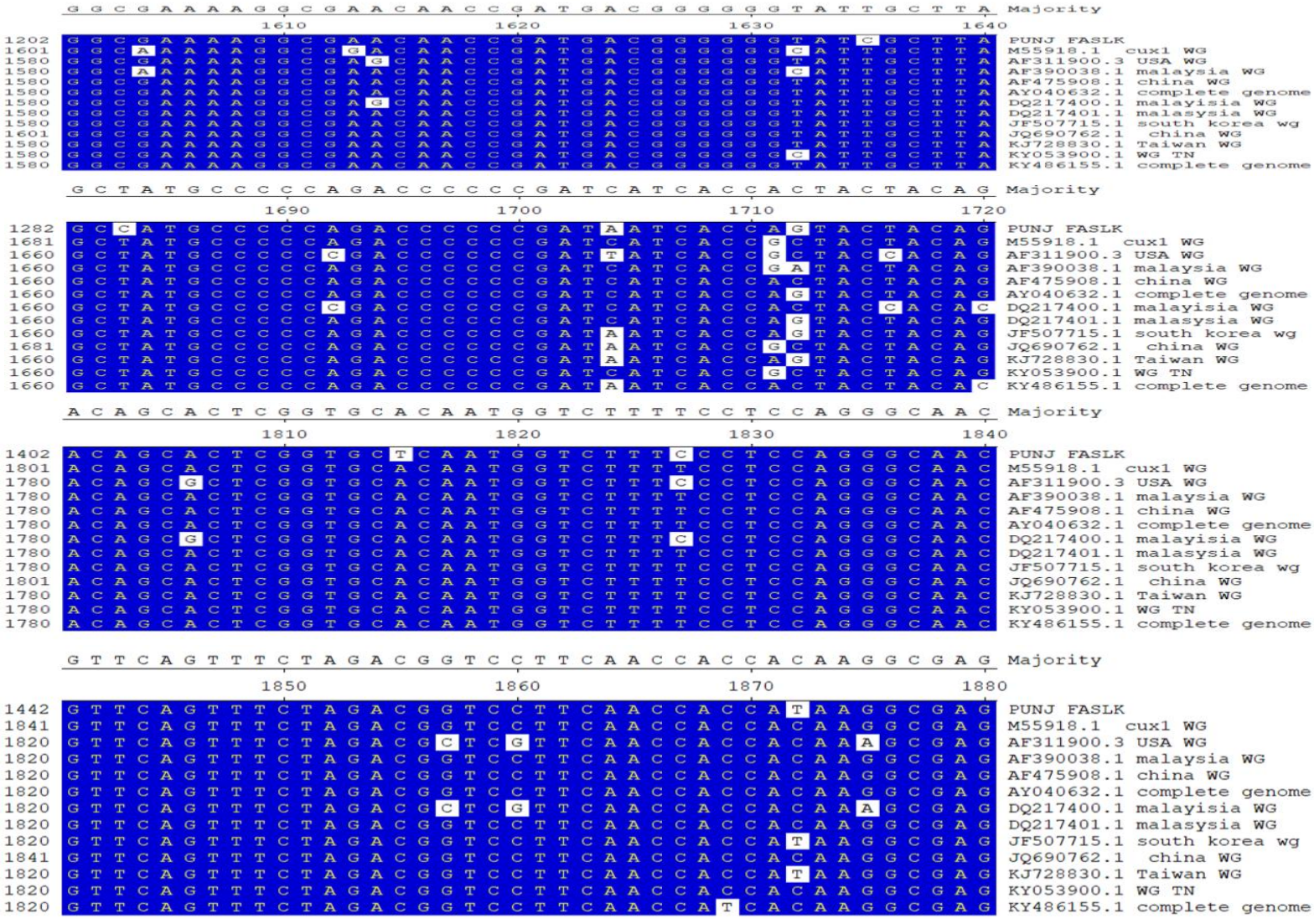
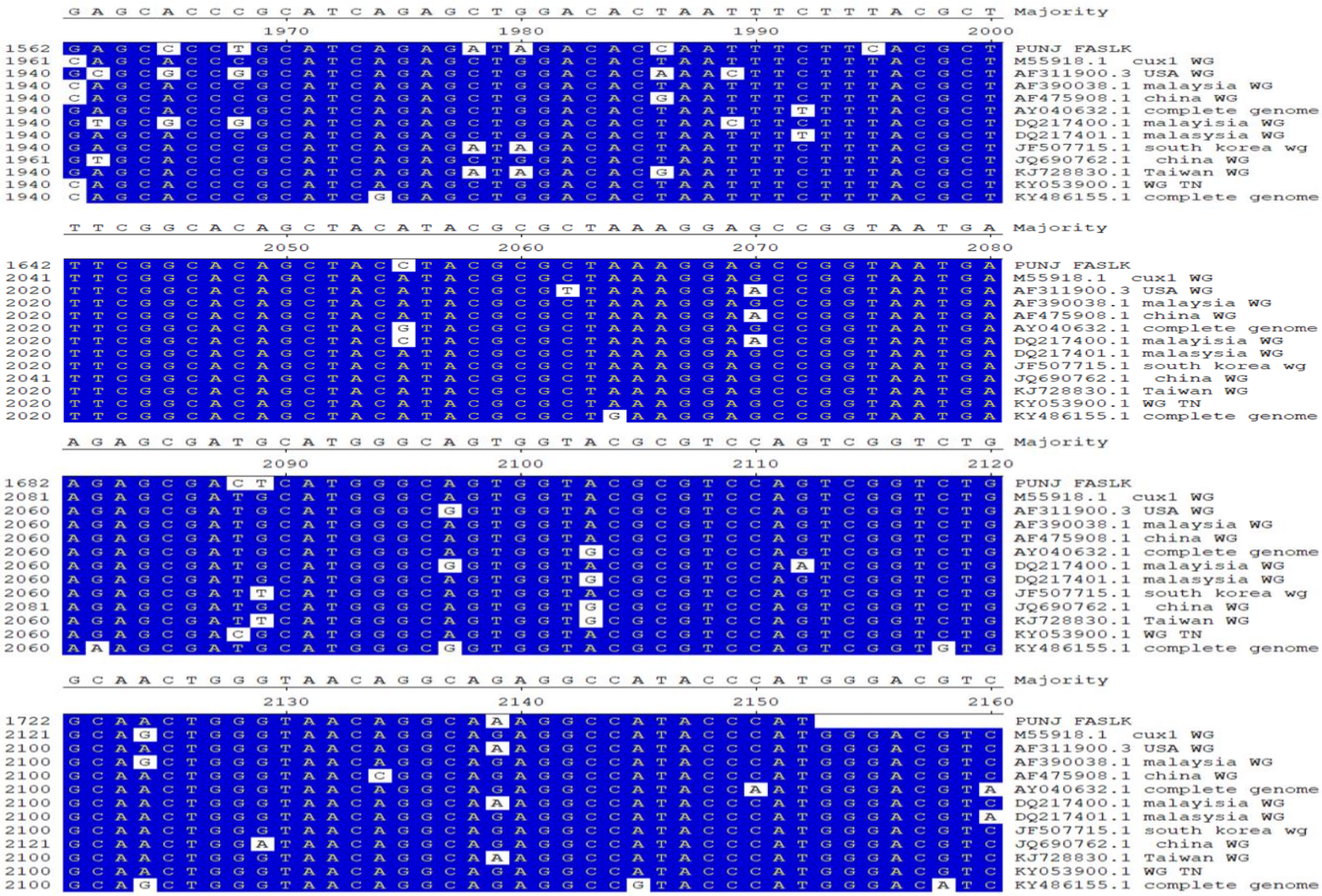

Majority
PUNJ FASLK
M55918.1 cux1 WG
AF311900.3 USA WG
AF390038.1 malaysia WG
AF475908.1 china WG
AY040632.1 complete genome
DQ217400.1 malayisia WG
DQ217401.1 malasysia WG
JF507715.1 south korea wg
JQ690762.1 china WG
KJ728830.1 Taiwan WG
KY053900.1 WG TN
KY486155.1 complete genome

Fig. 33 Contd..


G A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T C T T T A C G C T Majority
1970 1980 1990 2000
1562 G A G C C C T G C A T C A G A G A T A G A C A C C A A T T T C T T T C A C G C T PUNJ FASLK
1961 C A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T C T T T A C G C T M55918.1 cux1 WG
1940 G C G C G C C G G C A T C A G A G C T G G A C A C A A A C T T C T T T A C G C T AF311900.3 USA WG
1940 C A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T C T T T A C G C T AF390038.1 malaysia WG
1940 C A G C A C C C G C A T C A G A G C T G G A C A C G A A T T T C T T T A C G C T AF475908.1 china WG
1940 G A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T T T T T A C G C T AY040632.1 complete genome
1940 G T G C G C C G G C A T C A G A G C T G G A C A C T A A C T T C T T T A C G C T DQ217400.1 malayisia WG
1940 G A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T T T T T A C G C T DQ217401.1 malasysia WG
1940 G A G C A C C C G C A T C A G A G A T A G A C A C T A A T T T C T T T A C G C T JF507715.1 south korea wg
1961 G T G C A C C C G C A T C A G A G C T G G A C A C T A A T T T C T T T A C G C T JQ690762.1 china WG
1940 G A G C A C C C G C A T C A G A G A T A G A C A C G A A T T T C T T T A C G C T KJ728830.1 Taiwan WG
1940 C A G C A C C C G C A T C A G A G C T G G A C A C T A A T T T C T T T A C G C T KY053900.1 WG TN
1940 C A G C A C C C G C A T C G G A G C T G G A C A C T A A T T T C T T T A C G C T KY486155.1 complete genome
T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A Majority
2050 2060 2070 2080
1642 T T C G G C A C A G C T A C C T A C G C G C T A A A G G A G C C G G T A A T G A PUNJ FASLK
2041 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A M55918.1 cux1 WG
2020 T T C G G C A C A G C T A C A T A C G C G T T A A A G G A A C C G G T A A T G A AF311900.3 USA WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A AF390038.1 malaysia WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A A C C G G T A A T G A AF475908.1 china WG
2020 T T C G G C A C A G C T A C G T A C G C G C T A A A G G A G C C G G T A A T G A AY040632.1 complete genome
2020 T T C G G C A C A G C T A C C T A C G C G C T A A A G G A A C C G G T A A T G A DQ217400.1 malayisia WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A DQ217401.1 malasysia WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A JF507715.1 south korea wg
2041 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A JQ690762.1 china WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A KJ728830.1 Taiwan WG
2020 T T C G G C A C A G C T A C A T A C G C G C T A A A G G A G C C G G T A A T G A KY053900.1 WG TN
2020 T T C G G C A C A G C T A C A T A C G C G C T G A A G G A G C C G G T A A T G A KY486155.1 complete genome
A G A G C G A T G C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G Majority
2090 2100 2110 2120
1682 A G A G C G A C T C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G PUNJ FASLK
2081 A G A G C G A T G C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G M55918.1 cux1 WG
2060 A G A G C G A T G C A T G G G C G G T G G T A C G C G T C C A G T C G G T C T G AF311900.3 USA WG
2060 A G A G C G A T G C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G AF390038.1 malaysia WG
2060 A G A G C G A T G C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G AF475908.1 china WG
2060 A G A G C G A T G C A T G G G C A G T G G T G C G C G T C C A G T C G G T C T G AY040632.1 complete genome
2060 A G A G C G A T G C A T G G G C G G T G G T A C G C G T C C A A T C G G T C T G DQ217400.1 malayisia WG
2060 A G A G C G A T G C A T G G G C A G T G G T G C G C G T C C A G T C G G T C T G DQ217401.1 malasysia WG
2060 A G A G C G A T T C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G JF507715.1 south korea wg
2081 A G A G C G A T G C A T G G G C A G T G G T G C G C G T C C A G T C G G T C T G JQ690762.1 china WG
2060 A G A G C G A T T C A T G G G C A G T G G T G C G C G T C C A G T C G G T C T G KJ728830.1 Taiwan WG
2060 A G A G C G A C G C A T G G G C A G T G G T A C G C G T C C A G T C G G T C T G KY053900.1 WG TN
2060 A A A G C G A T G C A T G G G C G G T G G T A C G C G T C C A G T C G G T G T G KY486155.1 complete genome
G C A A C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C Majority
2130 2140 2150 2160
1722 G C A A C T G G G T A A C A G G C A A G G C C A T A C C C A T PUNJ FASLK
2121 G C A G C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C M55918.1 cux1 WG
2100 G C A A C T G G G T A A C A G G C A A G G C C A T A C C C A T G G G A C G T C AF311900.3 USA WG
2100 G C A G C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C AF390038.1 malaysia WG
2100 G C A A C T G G G T A A C C G G C A G A G G C C A T A C C C A T G G G A C G T C AF475908.1 china WG
2100 G C A A C T G G G T A A C A G G C A G A G G C C A T A C C A A T G G G A C G T A AY040632.1 complete genome
2100 G C A A C T G G G T A A C A G G C A A G G C C A T A C C C A T G G G A C G T C DQ217400.1 malayisia WG
2100 G C A A C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T A DQ217401.1 malasysia WG
2100 G C A A C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C JF507715.1 south korea wg
2121 G C A A C T G G A T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C JQ690762.1 china WG
2100 G C A A C T G G G T A A C A G G C A A G G C C A T A C C C A T G G G A C G T C KJ728830.1 Taiwan WG
2100 G C A A C T G G G T A A C A G G C A G A G G C C A T A C C C A T G G G A C G T C KY053900.1 WG TN
2100 G C A G C T G G G T A A C A G G C A G A G G C C G T A C C C A T G G G A C A T C KY486155.1 complete genome

CHAPTER – V

SUMMARY AND CONCLUSIONS

Chicken Infectious Anemia (CIA), a viral disease, causes severe immunosuppression in chicken and possesses potential to become a single cause of havoc for 22nd century poultry sector in India. The previous work done so far highlights an alarming situation for our poultry population which is very soon going to outnumber humans due to its phenomenal growth of over 5% per annum. Poultry is most organized sector among all the livestock species in India. Commercial chicken production is increasing in quantum according to rising appetite among Indians for non-vegetarian diet. Commercial or intensive chicken production involves rearing birds at higher density (less floor/cage space per bird) which puts stress on birds. Stress and Chicken Infectious Anemia virus is close ally in birds especially in younger ones leading to breaking the immune system of body and making birds prone to acquire secondary (other) infections as CIA virus shows synergy with some other viral diseases. These secondary infections are mostly fatal for these young birds and disguise the real culprit in post-mortem examination by making impression in pathologist's mind that birds are dying due to secondary infections. Symptoms of this viral infection are also similar to many other infections such as weakness, reduced growth, ruffled feathers, loss of appetite etc. leading to non-confirmation of CIA in the flock. Those lucky birds which survive or overcome this infection may bring havoc at farms in near future by becoming carrier. Carriers spread the virus inside the sheds through various secretions including feces. These viruses will enter in new host and flare up when birds will be under stress most often under summer/heat stress. Further, this virus also gets vertically transmitted (from parent stock to progeny through eggs) leading to rapid dissemination of the virus to the farms procuring chicks from infected breeder farms

Due to this reason despite having huge prevalence, impact of this disease is undermined in India and even vaccination is not usually recommended. Hence, it is a hidden billion-dollar virus for Indian poultry sector to catch. Therefore, present investigation was planned to explore the presence of CIA disease at poultry farms of Punjab state where poultry sector has grown to the tune of 57.17% in last five years.

The present study was planned to know the serological evidence, detection of CIAV circulating among the flocks and genetic characterization of isolated CIAV in poultry dense area of Punjab state. Serological study was conducted by indirect ELISA to detect the antibody to CIAV in the serum of chickens of various age group. Further, presence of virus in seropositive birds was detected by PCR and was genetically characterized. The presence of CIAV was also confirmed in tissues of dead birds which showed characteristic signs and symptoms of CIA. Further, isolation of the CIAV from field tissue samples was done in MDCC MSB1 cell line. The isolated virus was molecularly characterized and phylogenetic relationship was analyzed by various bioinformatic tools

In the sero-molecular study, chickens of various age group (n=120) from different flocks (n=27) and different geographical areas (n=12) were tested for evidence of seroconversion to CIAV. Majority of the sample was from apparently healthy birds (both layer and broiler) but few clinically ill birds also included in the present study. Sixty-six (55%) birds were found positive for anti-CIAV antibody. Fifty-one (56.04%) of the apparently healthy birds tested were also found positive for CIAV antibody.

The haematological profile of the birds under study was also corelated with serological study. There was significant decrease in packed cell volume, haemoglobin and erythrocyte count was observed among seropositive when compared to seronegative birds.

All seropositive birds screened for presence of CIAV genome by PCR and further sequenced to characterize CIAV circulating among the flocks. Majority of the birds (74.24%) were found positive for presence of CIAV genome. A significant change in white cell count was observed in haematology of these birds

Sequencing and bioinformatic analysis of the most conserved gene (VP3) showed 100% homology with various other reported Indian and world isolates of CIAV. There was no evidence of change or substitution at nucleotide and deduced amino acid sequence position in the VP3 gene coding apoptin protein. Phylogenetic relationship also agreed with these finding as there was no clear cut clad/cluster formation observed among the isolates included in the present study. Moreover, it was closely related to other Indian isolates.

The second part of the work was aimed to isolate CIAV from birds died of typical signs and symptoms of CIA. Tissue samples of dead birds (n=65) were collected from various regions (n=10) of Punjab state. Presence of virus was observed in almost all the lymphoid organs with highest was in thymus tissues. Thirty-nine (60%) birds were found positive for genome of CIAV by PCR method. Among the various age group, chicks of <20days were found highly positive for CIAV. But presence of virus in the group of 20 to 60 days also moderately positive for CIAV genome.

All the three important viral gene screened for confirming the amplified genome of CIAV also showed presence of virus in the dead birds. Further, each gene molecularly characterized and phylogenetic relation with other Indian isolates were compared. Among the three genes analyzed, VP1 gene showed nucleotide substitution at various positions but change in the nucleotides did not showed any obvious change in amino acid sequence of the virus. VP2 and VP3 gene were found to be highly conserved. Phylogenetic analysis showed close relationship with other Indian isolates reported.

Birds found positive for CIAV genome by PCR were further used for isolation of CIAV in cell line (MDCC MSB1). Ten isolates of CIAV were isolated from positive field samples. Growth of the virus was slow but could adapt to isolation condition. The cells infected with virus showed characteristic CPE of CIAV infection like ballooning, enlargement, aggregation of viral protein in the form of granules and finally rupturing and lysis of the cells. Six PCR positive field samples did not show any CPE in cell culture.

Confirmation of the cell culture isolate was done by immunoassays to demonstrate viral antigens and PCR for demonstration of CIAV genome. All the ten isolates were found positive for presence of CIAV antigen and genome.

The whole genome of the all cell culture isolates was amplified by PCR. It showed approximately 2.3Kb amplicon after resolving in electrophoresis. Three isolates were cloned in to prokaryotic vector and sequenced to characterize the whole genome of CIAV. Sequence obtained show high similarity with various Indian and global isolates reported. Point mutations were obvious in the genome of isolated CIAV. Deduced amino acid showed various substitution. No obvious cluster was

observed, but isolate from the study was closely clustered with isolates from Taiwan and south Korea.

Conclusions

The following conclusions could be drawn from the present study

- The CIAV is highly prevalent among chicken flocks of poultry dense Punjab state and presence of viral genome together with high antibody titre in apparently healthy bird indicate that virus is sub clinically circulating among various flocks
- Ten isolates of CIAV were isolated in cell culture from the field samples
- The isolates obtained in the present study is closely related to other isolates in India and in the world
- Molecular study and Phylogenetic analysis indicated that isolates of CIAV circulating in Punjab might have originated from various isolates from different parts of the India or world
- The isolates obtained in the present study lie in the same cluster with the isolates reported from the different parts of India

REFERENCES

AboElkhair M, El-Razak A G A and Metwally A E Y. 2014. Molecular characterization of chicken anemia virus circulating in chicken flocks in Egypt. *Advances in Virology* 797151.

Adair B M 2000. Immunopathogenesis of chicken anemia virus infection. *Developmental and. Comparative Immunology* **24**: 247-55.

Al-HassaniA S A. 2011. Effect of dietary supplementation with different levels ofarginine on some blood traits of laying hens. *International Journal of Poultry Science* **10**(9): 705-09.

Baksi S, Savaliya B F, Rao N and Khan M 2016. Sero-Prevalence of Chicken Anaemia Virus (CAV) in Layer Poultry Flocks in Different Parts of India. *Journal of Poultry Science*. **4(03):**39-42.

Balamurugan V and Kataria J M 2006. Economically important non-oncogenic immunosuppressive viral diseases of chicken - Current status. *Veterinary Research Communications***30**(5): 541–66.

Bennett C E, Thomas R, Williams M, Zalasiewicz J, Edgeworth M, Miller H, Coles B, Foster A, Burton E J and Marume U. 2018. The broiler chicken as a signal of a human reconfigured biosphere. *Royal Society Open Science* **5**:180325.

Bhatt P, Shukla S K, Mahendran M, Dhama K, Chawak M M and Kataria J M. 2011. Prevalence of chicken infectious anemia virus (CIAV) in commercial poultry flocks of northern India: A Serological Survey. *Transboundary and Emerging Diseases* **58**: 458-60.

Bhatt P, Shukla S K, Wani M Y, Tiwari R and Dhama K 2013. Amelioration of chicken infectious anaemia virus induced immunosuppression by immunomodulator and haematinic supplementation in chicks. *Veterinarski arhiv* **83**(6):639-52.

Biđin M, Savić V, Biđin Z, Balenović M and Majnarić D. 2010. The prevalence of antibodies against chicken anemia virus in unvaccinated broilers and broiler breeders in Croatia. *Veterinarski Arhiv* **80**: 753-60.

Brentano L, Lazzarina S, Bassia S S, Kleina T A P and Schat K A. 2005. Detection of chicken anemia virus in the gonads and in the progeny of broiler breeder hens with high neutralizing antibody titers. *Veterinary Microbiology* **105**: 65–72.

Brentano L, Mores N, Wentz I, Chandratilleke D and Schat K A. 1991. Isolation and Identification of Chicken Infectious Anemia Virus in Brazil. *Avian Diseases* **35**: 793-800.

Bülow V V and Fuchs B. 1986. Attenuierung des Erregers der aviären infektiösen Anämie (CAA) durch Serienpassagen in Zellkulturen. *Journal of Veterinary Medicine Series B* **33**:568–573.

Bulow V V and Schat K A. 1997. Chicken infectious anemia. In: Calnek B W, Barens H J, Beard C W, McDougald L R and Saif Y M (Eds.) *Disease of Poultry*, Iowa University Press pp: 736-56.

Canal C W, Ferreira D J, Macagnan M, Fallavena L C, Moraes H L and Wald V B 2004. Prevalence of antibodies against chicken anaemia virus (CAV) in broiler breeders in Southern Brazil. *Pesquisa Veterinária Brasileira.* **24(2)**:89-92.

Cardona C J, Oswald W B and Schat K A. 2000. Distribution of chicken anaemia virus in the reproductive tissues of specific pathogen-free chickens. *Journal of General Virology* **81**: 2067-75.

Cardona C J, Oswald W B and Schat K A. 2000. Distribution of chicken anaemia virus in the reproductive tissues of specific pathogen-free chickens. *Journal of General Virology*. **81**: 2067-2075.

Caterina K M, Frasca Jr S, Girshick T and Khan M I. 2004. Development of a multiplex PCR for detection of avian adenovirus, avian reovirus, infectious bursal disease virus, and chicken anemia virus. *Molecular and Cellular Probes* **18**: 293-98.

CAV.2016. Chicken anemia virus antibody ELISA. 1-14 http://www.asineh.com/PDF/Biochek_New/CAV%20datapack.pdf.

Chansiripornchai N, Pongthanes S, Chansiripornchai P and Wanasawaeng W. 2013. Development of Enzyme-Linked Immunosorbent Assay to detect antibodies against Chicken Infectious Anemia Virus. *Thai Journal of Veterinary Medicine* **43**(3): 353-57.

Chansiripornchai N. 2016. Field study of seroconversion of three different commercial vaccines of chicken infectious anemia virus in Thailand. *The Thai Journal of Veterinary Medicine* **46(4):**699.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson T J, Higgins D G and Thompson J D 2003. Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Research* **31**: 3497-3500.

Chowdhury S M Z H, Omar A R, Aini I, Hair-Bejo M, Jamaluddin A A, Md-Zain B M, and Kono Y. 2003. Pathogenicity, sequence and phylogenetic analysis of Malaysian chicken anaemia virus obtained after low and high passages in MSB-1 cells. *Archives of Virology* **148**:2437–2448.

Crowther R A, Berriman J A, Curran W L, Allan G M and Todd D. 2003. Comparison of the structures of three Circoviruses: Chicken Anemia Virus, Porcine Circovirus Type 2, and Beak and Feather Disease Virus. *Journal of Virology* **77:** 13036-41.

Cunningham S C, Lew A M and Tannock G A. 2001. The antigenicity of the chicken anaemia virusprotein VP3 (Apoptin). *Avian Pathology* **30:** 613-19.

Davidson I, Kedem M, Borochovitz H, Kass N, Ayali G, Hamzani E, Perelman B, Smith B, and Perk S. 2004. Chicken infectious anemia virus infection in Israeli commercial flocks: virus amplification, clinical signs, performance, and antibody status. *Avian Diseases* **48**:108–118.

De Herdt P, Van den Bosch G, Ducatelle R, Uyttebroek E Schrier C. 2001. Epidemiology and significance of chicken infectious anemia virus infections in broilers and broiler parents under nonvaccinated European circumstances. *Avian diseases*. **1**:706-8.

Dhama K, Kataria J M, Senthilkumar N, Tomar S and Dash B B. 2002. Standardization and application of polymerase chain reaction and indirect immunofluorescent techniques for detection of chicken anemia virus. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **23**:111-22.

Dhama K. 2002. Ph D Thesis. Submitted to Deemed University, Indian Veterinary Research Institute, Izatnagar, India.

Eltahir Y M, Qian K, Jin W and Qin A. 2011a. Molecular epidemiology of chicken anemia virus in commercial farms in China. *Virology Journal* **8**:145. doi: 10.1186/1743-422X-8-145

Eltahir Y M, Qian K, Jin W and Qin A. 2011b. Analysis of chicken anemia virus genome: evidence of intersubtype recombination. *Virology Journal* **8**: 512. doi: 10.1186/1743-422X-8-512

Emikpe B O, Oluwayelu D O, Ohore O G, Oladele O A and Oladokun A T. 2005. Serological evidence of chicken anemia virus infection in Nigerian indigenous chickens. *Onderstepoort Journal of Veterinary Research* **72**: 101-03.

Engström B E and Luthman M. 1984. Blue wing disease of chickens: Signs, pathology and natural transmission. *Avian Pathology* **13**:1

Erfan A M, Abdullah A S and Naguib M M. 2018. Characterization of full genome sequences of chicken anemia viruses circulating in Egypt reveals distinct genetic diversity and evidence of recombination. *Virus Research* **251**: 78-85. https://doi.org/10.1016/j.virusres.2018.05.008

Farhoodi M, Toroghi R, Bassami M R and Kianizadeh M. 2007. Chicken infectious anaemia virus infection among broiler chicken flocks in Iran. *Archives of Razi Institute* **62**:1-6

Goldsmith C S, Ksiazek T G, Rollin P E, Comer J A, Nicholson W L, Peret T C, Erdman D D, Bellini W J, Harcourt B H, Rota P A, Bhatnagar J, Bowen M D, Erickson B R, McMullan L K, Nichol S T, Shieh W J, Paddock C D. and Zaki S R. 2013. Cell culture and electron microscopy for identifying viruses in diseases of unknown cause. *Emerging Infectious Diseases* **19**(6): 886-91.

Goryo M, Sugimura H, Matsumoto S, Umemura T, and Itakura C. 1985. Isolation of an agent inducing chicken anaemia. *Avian Pathology* **14**:483–496.

Goryo M, Suwa T, Matsumoto S, Umemura T and Itakura C. 1987. Serialpropagation and purification of chicken Anaemia agent in mdcc-MSB1 cell line. *Avian Pathology* **16**: 149-163. DOI: 10.1080/03079458708436360

Goryo M, Suwa T, Umeura T, Itakura C and Yamashiro S. 1989. Histopathology of chicks inoculated with chicken anemia agent (MSB1-TK 5803 strain). *Avian Pathology* **18**: 73-89.

Goryo, M, Suwa T, Matsumoto S, Umemura T, and Itakura C. 1987. Serial propagation and purification of chicken anaemia agent in MDCC-MSB1 cell line. *Avian Pathology* 16:149–163

Gowthaman V, Singh S D, Dhama K, Barathidasan R, Srinivasan P, Mahajan N K and Ramakrishnan M A. 2014. Molecular characterization of chicken anemia virus isolated from commercial poultry with respiratory disease complex in India. *Advances in Animal and Veterinary Sciences* **2(3):** 171-76.

Hadimli H H, Erganiş O, Güler L and Uçan U S 2008. Investigation of chicken infectious anemia virus infection by PCR and ELISA in chicken flocks. *Turkish Journal of Veterinary and Animal Sciences*. 32(2):79-84.

Hailemariam Z, Omar A R, Hair-Bejo M and Giap T C. 2008. Detection and characterization of chicken anemia virus from commercial broiler breeder chickens. *Virology Journal*. **5**: 128

Hiremath C, Jhala M K, Bhanderi B B and oshi C G 2013. Cloning and sequence analysis of VP1, VP2 and VP3 genes of Indian chicken anemia virus. *Iranian Journal of Veterinary Research*. **14(4):**354-7.

Hussein H A, Sabry M Z, El Ibiary E A, El Safty M and Abd El Hady A I. 2002. Chicken infectious anemia virus in Egypt: Molecular diagnosis by PCR and isolation of the virus from infected flocks. *Arab Journal of Biotechnology* **5**(2): 263-74.

ICTV 2011. Virus Taxonomy (Classification and Nomenclature of Viruses). The online (9^{th}) report of the International Committee on Taxonomy of Viruses.

ICTV 2017. Virus Taxonomy (Classification and Nomenclature of Viruses). The online (10^{th}) report of the International Committee on Taxonomy of Viruses.

Islam M R, Johne R, Raue R, Todd D and Muller H. 2002. Sequence Analysis of the Full-Length Cloned DNA of a Chicken Anaemia Virus (CAV) Strain from Bangladesh: Evidence for Genetic Grouping of CAV Strains Based on the Deduced VP1 Amino Acid Sequences. *Journal of Veterinary Medicine Series B* **49**(7): 332–37.

Iwata N, Fujino M, Tuchiya K, Iwata A, Otaki Y and Ueda. 1997. Development of an enzyme linked immunosorbent assay using recombinant chicken anemia virus proteins expressed in a baculovirus vector system. *Journal of Veterinary Medical Science* **60**(2): 175-80.

Jaffe P. 1960. Differences in numbers of erythrocytes between inbred lines of chickens. *Nature* **186**:978–979.

Jeurissen S H, Wagenaar F, Pol J M, van der Eb A J and Noteborn M H. 1992. Chicken anemia virus causes apoptosis of thymocytes after *in vivo* infection and of cell lines after in vitro infection. *Journal of Virology* **66**(12): 7383-88.

Johnson K M, Lange J V, Webb P A and Murphy F A. 1977. Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. *Lancet* **1**: 569–71.

Kataria J M, Mohan M C, Dey S, Dash B B and Dhama K. 2005. Diagnosis and immune prophylaxis of economically important poultry diseases: a review. *Indian Journal of Animal Sciences* **75**(5): 555-67.

Kataria J M, Suresh R P, Verma K C, Toroghi R, Kumar N S, Kataria R S and Sah R L. 1999. Chicken infectious anaemia (CIA) in India: detection of the agent bypolymerase chain reaction and transmission study. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **20**: 91-95.

Kato A, Fujino M, Nakamura T, Ishihama A and Otaki Y. 1995. Gene organization of chicken anemia virus. *Virology* **209**: 480-88.

Khoshkhoo P H, Akbariazad G and Tashakori M 2011. Comparison of CAV antibody titers in a vaccinated and naturally infected broiler breeder flocks. *African Journal of Microbiology Research.* **5(20):**3162-5.

Kim H R, Kwon Y K, Bae, Y C, Oem J K and Lee O S. 2010. Molecular characterization of chicken infectious anemia viruses detected from breeder and broiler chickens in South Korea. *Poultry Science* **89**: 2426–31.

Koch G, van Roozelaar D J, Verschueren C A, van der Eb A J and Noteborn M H. 1995. Immunogenic and protective propertiesof chicken anaemia virus proteins expressed by baculovirus. *Vaccine* **13**: 763–70.

Krishan G, Shukla S K, Bhatt P, Kumar R, Tiwari R, Malik Y S, and Dhama K. (2015b). Immunomodulatory and therapeutic prospective of a protein supplement with vitamins and selenium (Multimune) against chicken infectious anaemia in broiler chicks. *Advances in Animal and Veterinary Sciences* **3(3s):** 1-8.

Krishan G, Shukla S K, Bhatt P, Wani M Y, Dhama K, Malik Y P S and Kumar R. 2015. Clinical association of chicken anemia virus with other infectious poultry diseases in north India and Nepal: its pathological studies, molecular epidemiology and RFLP Pattern of PCR amplified full length viral genome. *Advances in Animal and Veterinary Sciences* **3**(7): 395-405.

Kumar S, Stecher G and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* **33**(7): 1870-74.

Kye S J, Kim J Y, Seul H J, Kim S, Kim S E, Lee H S, Sorn S and Choi K S. 2013. Phylogenetic analysis and genetic characterization of chicken anemia virus isolates from Cambodia. *Poultry Science* **92**: 2681-86.

Lambden P R, Cooke S J, Caul E O and Clarke I N. 1992. Cloning of non cultivable rotavirus by single primer amplification. *Journal of Virology* **66**: 1817-22.

Latheef S K, Dhama K, Wani M Y, Samad H A, Tiwari R and Singh S D 2013. Ameliorative effects of Withania somnifera, Azadirachta indica, Tinospora cordifolia and E care Se herbal preparations on chicken infectious anaemia virus induced haematological changes in chicks and their live body weights. *South Asian Journal of Experimental Biology*. **3**(4):172-82.

Ledesma N, Fehervari T, Casaubon M T, Lucio E and Ratz F. 2001. Chicken Infectious Anemia in Mexico: Virus Identification and Serology Survey. *Avian Diseases* **45**(4): 788.

Lee M S, Hseu Y C, Lai G H, Chang W T, Chen H J, Huang C H, Lee M S, Wang M Y, Kao J Y, You B J, Lin W H, Lien Y Y and Lin M K. 2011. High yield expression in a recombinant *E. coli* of a codon optimized chicken anemia virus capsid protein VP1 useful for vaccine development. *Microbial Cell Factories* **10**:56.

Lee M, Lien Y, Feng S, Huang R, Tsai M, Chang W and Chen H. 2008. Production of chicken anemia virus (CAV) VP1 and VP2 protein expressed by recombinant *Escherichia coli*. *Process Biochemistry* **44**: 390-95.

Li Y, Fang L, Cui S, Fu J, Li X, Zhang H, Cui Z, Chang S, Shi W and Zhao P. 2017. Genomic Characterization of Recent Chicken Anemia Virus Isolates in China. *Frontiers in Microbiology* **8**: 401.

Li Y, Wang Y, Fang L, Fu J, Cui S, Zhao Y, Cui Z, Chang S and Zhao P. 2016. Genomic analysis of the chicken infectious anemia virus in a specific pathogen-free chicken population in China. BioMed Research International 2016: 4275718

Livestock census 2012. 19thLivestock census- 2012 all India report. Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Krishi Bhawan, New Delhi. http://dahd.nic.in/sites/default/filess/Livestock%20%205_0.pdf

Lucio B, Schat K A and Shivaprasad H L. 1990. Identification of the CAA, reproduction of the disease and serological survey in the United States. *Avian Diseases* **34**: 146-53.

Mahzounieh M, Karimi I and Zahraei S T 2005. Serologic evidence of chicken infectious anemia in commercial chicken flocks in Shahrekord, Iran. *International Journal of Poultry Science* **4(7):**500-3.

McConnell C D, Adair B M and McNulty M S. 1993. Effects of chicken anemia virus on cell-mediated immune function in chickens exposed to the virus by a natural route. *Avian Diseases* **37**: 366-74.

McIlroy S G, McNulty M S, Bruce D W, Smyth J A, Goodall E A and Alcorn M J 1992. Economic effects of clinical chicken anemia agent infection on profitable broiler production. *Avian diseases* **1**:566-74.

McNulty M S, Connor T J, McNeilly F and Spackman D. 1989. Chicken anemia agent in the United States: isolation of the virus and detection of antibody in broiler breeder flocks. *Avian Diseases* **33**: 691-94.

McNulty M S, Connor T J, McNeilly F, Kirkpatrick K S, and McFerran J B. 1988. A serological survey of domestic poultry in the United Kingdom for antibody to chicken anemia agent. *Avian Pathology* **17**: 315-24.

McNulty M S, Curran W L, Todd D and Mackie D P. 1990. Chicken Anemia Agent: An Electron Microscopic Study. *Avian Diseases* **34**(3): 736-43.

McNulty M S. 1991. Chicken anemia agent: A review. *Avian Pathology* **20**(2): 187-203.

Meehan B M, Todd D, Creelan J L, Connor T J and McNulty M S. 1997. Investigation of the attenuation exhibited by a molecularly cloned chicken anemia virus isolate by utilizing a chimeric virus approach. *Journal of Virology* **71**: 8362-67.

Miles M, Reddy M and Morgan W. 2001. Co-infection of specific-pathogen-free chickens with Marek's disease virus (MDV) and chicken infectious anemia virus: effects of MDV pathotype. *Avian Diseases* **45**: 9-18.

Miller M M and Schat K A. 2004. Chicken Infectious Anemia Virus: An Example of the Ultimate Host–Parasite Relationship. *Avian Diseases* **48**(4): 734-45.

Miller M M, Ealey K A, Oswald W B and Schat K A. 2003. Detection of chicken anemia virus DNA in embryonal tissues and eggshell membranes. *Avian Diseases*. **47(3):** 662-671.

Mohammadreza K I, Shahab M and Azad B F. 2010. Chicken anemia virus infection in broiler chickens in Shahrekord, Iran: serological, hematological, and histopathological findings. *Comparative Clinical Pathology* **19**: 63-67.

Narayani and Ghosh RC. 2018. Studies on Pathogenicity of Chicken Infectious Anaemia Virus Isolated from a Poultry Flock of Chhattisgarh, India. *Journal of Animal Research.* **8**: 3

Natesan S, Kataria J M, Dhama K, Rahul S and Baradhwaj N. 2006. Biological and molecular characterization of chicken anaemia virus isolates of Indian origin. *Virus Research* **118**(1-2): 78–86.

Natt M P and Herrick C A. 1952. A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poultry Science* **31**: 735-38.

Nayabian H and Karim M. 2013. Molecular characterization of the chicken anaemia viruses isolated from broiler farms of west Azerbaijan, Iran. *Avian Pathology* **42**(2): 108-113.

Nogueira E O, Brentano L and Ferreira A J. 2005. A VP3/VP1 gene polymerase chain reaction assay for detection of chicken anemia virus in broiler samples. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia.* **57:**131-40.

Noteborn M H M and Koch G. 1995. Chicken anemia virus infection: molecular basis of pathogenicity. *Avian Pathology* **24**: 11-31.

Noteborn M H M, Todd D, Verschueren C A J, De Gauw H W F M, Curran W L, Veldkamp S, Douglas A J, McNulty M S, Van Der Eb AJ and Koch G. 1994. A single chicken anemia virus protein induces apoptosis. *Journal of Virology* **68**: 346-51.

Noteborn M H M, Verschueren C A J, Koch G and Van Der Eb A J. 1998. Simultaneous expression of recombinant baculovirus-encoded chicken anaemia virus (CIAV) proteins VP1 and VP2 is required for formation of the CIAV-specific neutralising epitope. *Journal of General Virology* **79**: 3073-77.

Noteborn M H M. 2004. Chicken anemia virus induced apoptosis: underlying molecular mechanisms. *Veterinary Microbiology* **98**(2): 89-94.

Noteborn M H M., De Boer G F, Roozelaar V, Karreman D J, Kranenburg C, Jeurissen O, Hoeben S H M, Zantema R C, Koch A, Van Ormondt G H and Van Der Eb A J. 1991. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *Journal of Virology* **65**: 3131-39.

Novak R and Ragland W L. 1997. *Insitu* hybridization for detection of chicken anaemia virus in peripheral blood smears. Molecular and cellular probes. **11**(2):135-41.

Olszewska-Tomczyk M, Świętoń E, Minta Z and Śmietanka K. 2016. Occurrence and phylogenetic studies of chicken anemia virus from Polish broiler flocks. *Avian Diseases* **60**(1):70-74.

Oluwayelu D O, Todd D and Olaleye O D. 2008. Sequence and phylogenetic analysis of chicken anemia virus obtained from backyard and commercial chickens in Nigeria. *Onderstepoort Journal of Veterinary Research* **75**(4): 353-57.

Oluwayelu D O. 2010. Diagnosis and epidemiology of chicken infectious anemia in Africa. *African Journal of Biotechnology* **9**(14): 2043-49.

Otaki Y, Saito K, Tajima A, and Nomura Y. 1991. Detection of antibody to chicken anaemia agent: a comparison of three serological tests. *Avian Pathology* **20**:315–324.

Ou S C, Lin H L and Liu P C. 2018. Epidemiology and molecular characterization of chicken anaemia virus from commercial and native chickens in Taiwan. *Transboundary and Emerging Diseases* **65**: 1-9.

Owoade A A, Oluwayelu D O, Fagbohun O A, Ammerlaan W, Mulders M N, Muller C P. 2004. Serologic evidence of chicken infectious anemia in commercial chicken flocks in Southwest Nigeria. *Avian Diseases* **48**: 202-05.

Pope C R. 1991. Chicken anemia agent. *Veterinary Immunology and Immunopathology* **30**:51–65.

Prajapati K S. (2010). Pathological and differential cytokine expression study in natural outbreak of chicken infectious anaemia virus infection. Doctoral dissertation, *Anand Agricultural University*, Anand.

Praveen B N, Dhama K, Kataria J M, Dash B B and Singh S D 2008. Detection and isolation of Chicken Infectious Anaemia Virus (CIAV) from field samples of poultry flocks from Gujarat and Andhra Pradesh, India. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases. **29**:23-6.

Renshaw R W, Soine C, Weinkle T, O'Connell P H, Ohashi K, Watson S, Lucio B, Harrington S and Schat K A. 1996. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *Journal of Virology* **70**: 8872-78.

Rosario K, Breitbart M, Harrach B, Segalaes J, Delwart E, Biagini P and Varsani A. 2017. Revisiting the taxonomy of the family Circoviridae: Establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Archives of Virology* **162**: 1447-63.

Rosenberger J K and Cloud S S. 1989a. The isolation and characterization of chicken anemia agent (CAA) from broilers in the United States. *Avian Diseases* **33**(4): 707-13.

Rosenberger J K and Cloud S S. 1989b. The effects of age, route of exposure, and co-infection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian Diseases* **33**: 753-59.

Rosenberger J K and Cloud S S. 1998. Chicken Anemia Virus. *Poultry Science* **77**: 1190-92

Saini N S and Dandapat A. 2009. Diagnosis and molecular characterization of chicken anemia virus. *Veterinary World* **2**(4):156-60.

Samy A and Naguib M M. 2018. Avian Respiratory Coinfection and Impact on Avian Influenza Pathogenicity in Domestic Poultry Field and Experimental Findings. *Veterinary Science* **5**: 23.

Santen V V L, Li L, Hoerr F J and Lauerman L H. 2001.Genetic characterization of chicken anemia virus from commercial broiler chickens in Alabama. *Avian Diseases* **45**: 373-88.

Sawant P M, Dhama K, Rawool D B, Wani M Y, Tiwari R, Singh S D and Singh R K. 2015. Development of a DNA vaccine for chicken infectious anemia and itsimmunogenicity studies using high mobility group box 1 protein as a novel immunoadjuvant indicated induction of promising protective immune responses. *Vaccine* **33**: 333–40.

Schat K A and van Santen V L. 2013. Chicken infectious anemia. In D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, & V. Nair (Eds.), Disease of poultry, 13th ed. (pp. 248–264). Ames, IA: John Wiley & Sons Inc.

Schat K A. 2003. Chicken infectious anemia. In Y.M. Saif (Ed.), Diseases of Poultry, 11th edn (pp. 182 202). Ames: Iowa State Press.

Sharma R N, Tiwari K, Chikweto A, Thomas D and Stratton G 2014. Serological evidence of chicken infectious anemia in layer and broiler chickens in Grenada, West Indies, *Veterinary World* **7(2):**59-61

Shettima Y M, El-Yuguda A D, Oluwayelu D O, Abubakar M B, Hamisu T M, Zanna M Y, maunta Maina M, Andrew A and Baba S S. 2017. Seroprevalence of chicken infectious anemia virus infection among some poultry species in Maiduguri, Nigeria. *Journal of Advanced Veterinary and Animal Research.* **4(4):**385-9.

Shulman L M and Davidson I. 2017. Viruses with Circular Single-Stranded DNA Genomes are everywhere! *Annual Review of Virology* **4**(1): 159-80.

Simeonov K B, Petrova R T, Gyurov B I, Peshev R D and Mitov B K. 2014. Isolation and PCR identification of chicken anemia virus infection in Bulgaria. *Bulgarian Journal of Veterinary Medicine* **17**(4): 276-84.

Simionatto S, Lima-Rosa C A, Binneck E, Ravazzolo A P and Canal C W. 2006. Characterization and phylogenetic analysis of Brazilian chicken anaemia virus. *Virus Genes* **33**: 5-10.

Smock C J, Komoyo G F, Mbee B P, Nakouné E, Faou A L, Okwen M P and Muller C P 2012. Epidemiology of chicken anemia virus in Central African Republic and Cameroon. *Virology Journal* **9**:189.

Smyth J A, Moffett D A, Connor T J and McNulty M S. 2006. Chicken anemia virus inoculated by the oral route causes lymphocyte depletion in the thymus in 3-weekold and 6-week-old chickens. *Avian Pathology* **35**(3): 254-59.

Smyth J A, Moffett D A, McNulty M S, Todd D and Mackie D P. 1993. A sequential histopathologic and immunocytochemical study of chicken anemia viurs infection at one day of age. *Avian Diseases* **37**: 324-38.

Spackman E, Cloud S S and Rosenberger J K. 2002b.Comparison of a putative second serotype of chicken infectious anemia virus with a prototypical isolate II. Antigenic and physicochemical characteristics. *Avian Diseases* **46**: 956-63.

Spackman E, Cloud S S, Pope C R and Rosenberger J K. 2002a. Comparison of a putative second serotype of chicken infectious anemia virus with a prototypical isolate I. Pathogenesis. *Avian Diseases* **46**: 945–55.

Subramanyam K V, Purushothama V, MuraliManohar B, Ravikumar G and Manoharan S. 2010. Development of Dot – Enzyme Linked Immunosorbent Assay for Detection of Antibodies to Infectious Bursal Disease, Hydro pericardium Syndrome and Chicken Anaemia Viruses. *EJBS* **4**(1): 08-18.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**: 2731-39.

Taniguchi T, Yuasa N, Maeda M and Horiuchi T. 1983. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. *National Institute of Animal Health Quarterly (Japan)* **23**:1–12.

Tham K M and Stanislawek W L. 1992. Polymerase chain reaction amplification for direct detection of chicken anemia virus DNA in tissues and sera. *Avian diseases*. 1:1000-6.

Todd D, Alistair N J, Scott N W, Ball B J, Borghmans and Brian M A. 2002. Molecular basis of the Attenuation Exhibited by Molecularly Cloned Highly Passaged Chicken Anemia Virus Isolates. *Journal of Virology* **76**: 8472-74

Todd D, Connor T J, Calvert V M, Creelan J L, Meehan B M and McNulty M S. 1995. Molecular cloning of an attenuated chicken anemia virus isolate following repeated cell culture passage. *Avian Pathology* **24**: 171-87.

Todd D, Creelan J L, Mackie D P, Rixon F and McNulty M S. 1990. Purification and biochemical characterization of chicken anaemia agent. *Journal of General Virology* **71**: 819–23.

Todd D, Douglas A J, Phenix K V, Curran W L, Mackie D P and McNulty M S. 1994. Characterization of chicken anemia virus. *In:* Proceedings, Int. Symp. On Infectious Bursal Disease and Chicken Infectious Anaemia (Ed. Kaleta, E.F.), Rauischholzhausen, Germany, 21-24 June, 1994, pp: 349-63.

Todd D, Mawhinney K A and McNulty M S. 1992. Detection and differentiation of chicken anemia virus isolates by using the polymerase chain reaction. *Journal of Clinical Microbiology* **30**:1661-66.

Todd D, Mawhinney K A, Graham D A and Scott A N J. 1999. Development of a blocking enzyme-linked immunosorbent assay for the serological diagnosis of chicken anemia virus. *Journal of Virological Methods* **82**(2): 177-84.

Todd D. 2000. Circoviruses: Immunosuppressive threats to avian species: A review. *Avian Pathology* **29**(5):373-94.

Toro H, Ewald S and Hoerr F J. 2006. Serological evidence of chicken infectious anemia virus in the United States at least since 1959. *Avian Dis*eases **50**(1):124-26.

Trinh Dai Q, Haruko O, Vuong N B, Tugsbaatar B, Mugimba K K, Shigeo Y and Kunitoshi I. 2015. Characterization of mAbs to chicken anemia virus and epitope mapping on its viral protein, VP1. *Journal of General Virology* **96**: 1086-97.

Vachhani K V. 2005. Etio-immuno-pathological studies on chicken infectious anemia-gangrenous dermatitis syndrome in pullets. Doctoral dissertation, *Anand Agricultural University*, Anand

Vagnozzi A E, Espinosa R, Cheng S, Brinson D, O'kane P, Wilson J and Zavala G. 2018. Study of dynamic of Chicken Infectious Anemia Virus (CIAV) Infection: Which sample is more reliable for viral detection? *Avian Pathology* **47**(5): 489-96.

Varmaghany S, Torshizi M A K, Rahimi S, Lotfollahian H and Hassanzadeh M. 2015. The effects of increasing levels of dietary garlic bulb on growth performance, systolic blood pressure, hematology, and ascites syndrome in broiler chickens. *Poultry Science* **94**:1812-20.

Vaziry A, Silim A, Bleau C, Frenette D and Lamontagne L. 2011. Chicken infectious anaemia vaccinal strain persists in the spleen and thymus of young chicks and induces thymic lymphoid cell disorders. *Avian pathology***. 40(4):**377-85.

Venugopalan A T, Elankumaran S, Raj G D, Manohar B M and Thangavelu A. 1994. Isolation of chicken anemia virus in Tamil Nadu. *Indian Veterinary Journal* **71**: 411-12.

Wang X Y, Wang X M, Gao Y L, Gao H L and Lu G L2006. Expression of the VP2 gene of chicken infectious anemia virus in E. coli and analysis of immunogenicity. *Wei sheng wu xue bao= Acta microbiologica Sinica.* **46**(5):841-3.

Wani M Y, Dhama K, Barathidasan R, Gowthaman V, Tiwari R, Bhatt P, Mahajan N K, Chawak M M, Singh S D and Kataria J M. 2013. Molecular detection and epidemiology of chicken infectious anemia virus in India. *South Asian Journal of Experimental Biology* **3**(4): 145-51.

Wani M Y, Dhama K, Latheef S K, Barathidassan R, Tiwari R, Chakraborty S, Chawak M M and Singh S D. 2014. Experimental Pathological Studies of an Indian Chicken Anaemia Virus Isolate and its Detection by PCR and FAT. *Pakistan Journal of Biological Sciences* **17**(6): 802-11.

Wani M Y, Dhama K, Tiwari R, Barathidasan R, Malik Y S, Singh S D, Singh R K 2015. Immunosuppressive effects of chicken infectious anaemia virus on T lymphocyte populations using flow cytometry and hematological parameters during experimental subclinical infection in chicks. *Advances in Animal and Veterinary Sciences* **3**(3): 143-150.

Wani M Y. 2013. Thesis. Development of recombinant protein Based elisa and real time-pcr for the Diagnosis of chicken anemia virus Infections in poultry. Indian Veterinary Research Institute, Izatnagar, India.

Yamaguchi S T, Imada N K, Masaji M, Kenji T, Tanimura N and Yuasa N. 2001. Identification of a genetic determinant of pathogenicity inchicken anaemia virus. *Journal of General Virology* **82**: 1233-38.

Yuasa N and Imai K. 1986. Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). *Avian Pathology* **15**: 639-45.

Yuasa N, Taniguchi T and Yoshida I. 1979. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Dis*eases **23**: 366-85.

Yuasa N. 1983. Propagation and infectivity titration of the Gifu-I s & w of chicken anemia agent in a cell line (MDCC-MSBI) derived from Marek's disease lymphoma. *National Institute of Animal Health Quarterly* **23**:13-20.

Zhang X, Liu Y, Wu B, Sun B, Chen F, Ji J, Ma J and Xie Q. 2013. Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. *Scientific Reports* **3**: 3519.

VITA

Name of the student	:	Dr. Sreekala S. Mohandas
Father's name	:	Mr. S. N. Mohandas
Mother's name	:	Mrs. P. K. Vijayakumari
Spouse	:	Dr. Sandeep Kaswan
Nationality	:	Indian
Date of Birth	:	07.05.1984
Permanent Home Address	:	I-75, Sachivalaya, Hanumangarh Junction Tehsil, Hanumangarh (D.T.), Rajasthan.

EDUCATIONAL QUALIFICATION

Bachelor's degree	**:**	**B.V.Sc. &A.H.**
University	:	College of Veterinary and Animal Science, Mannuthy, Kerala Agricultural University, Kerala
OGPA	:	7.30/10.00
Year of Award	:	2008
Master's Degree	**:**	**M.V.Sc. in Veterinary Virology**
University	:	Indian Veterinary Research Institute, Izatnagar, UP
OCPA	:	8.17/10
Year of Award	:	2013
Title of Thesis	:	Development of Loop Mediated Isothermal Amplification assay for rapid detection of Bluetongue virus
Ph.D.	**:**	**Veterinary Microbiology**
OCPA	:	8.34/10.00
Members of Societies	:	■ Member of Punjab State Veterinary Council ■ Life member of ISVBI ■ Life member of IVS
Awards/Distinction/ Fellowship/Scholarship	:	■ I.V.R. I institutional Fellowship during M. V. Sc. Programme ■ NET (ICAR-ASRB) for lectureship 2014 ■ 6th rank in AICE-SRF(PG) exam 2016 ■ GADVASU merit scholarship during 2016 ■ DST-Women Scientist-A (WOS-A) fellowship during 2017 onwards