INVESTIGATION ON FOWL ADENOVIRUS (GROUP 1) CIRCULATING IN BANGLADESH AND MOLECULAR CHARACTERIZATION OF SPECIFIC SEROTYPES ASSOCIATED WITH INCLUSION BODY HEPATITIS

PhD Dissertation

DEPARTMENT OF MICROBIOLOGY

FACULTY OF VETERINARY AND ANIMAL SCIENCE

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

June, 2024

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A Dissertation

Submitted By

Mohammad Nazrul Islam

Registration No.: 1205017

Session: 2020

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology

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Approved as to style and content by

Prof. Dr. Md. Mostafizer Rahman	**Dr. Khalesur Rahman**
Research Supervisor	**Research Co-Supervisor**

Prof. Dr. Md. Khaled Hossain

Chairman of the Examination Committee

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Preface

The dissertation entitled "**Investigation on Fowl adenovirus (group 1) circulating in Bangladesh and Molecular characterization of specific Serotypes associated with Inclusion Body Hepatitis"** is an outcome of the research study conducted during 2020 to 2023 at the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for partial fulfillment of the requirements for the degree of Doctor of Philosophy. The research project for the PhD study was partially supported by the Institute of Research and Training (IRT), HSTU.

Although FAdV infection was suspected, there was no confirmatory report from Bangladesh. The major intervention areas of the research study were to investigate the FAdV infection clinically in broiler, breeder & commercial layer, detection of antibodies in blood serum of infected flocks, molecular detection of viruses by real-time PCR and conventional PCR, sequencing of PCR products for serotyping and phylogenetic analysis. These findings indicated several introductions of FAdVs & specific serotypes associated with Inclusion Body Hepatitis in Bangladesh.

It is expected that the outcome of this PhD research study would assist to combat the disease by vaccination against detected specific serotypes causing IBH (Inclusion Body Hepatitis) along with maintenance of a comprehensive biosecurity measure which would influence the profitability & sustainability of our poultry industry across the country.

Mohammad Nazrul Islam

June, 2024

Declaration

The author wrote and composed this dissertation. It is a record of the author's original research work conducted under the supervision of Professor Dr. Md. Mostafizer Rahman and Co-supervisor Dr. Md. Khalesur Rahman. The PhD study was partially funded by the Institute of Research and Training (IRT), HSTU under the project **"Investigation on Fowl adenovirus (group 1) circulating in Bangladesh and Molecular characterization of specific Serotypes associated with Inclusion Body Hepatitis"** and was conducted at the Serology & Molecular Laboratory of the Department of Microbiology, HSTU, Dinajpur, and the Animal Health Section, National Institute of Biotechnology, Savar, Dhaka. This dissertation is the result of original research work, except where the references are included for contributions of others. All sources of such information are listed in the reference section. All forms of assistance and support are much appreciated and thanked.

None of the work has been submitted anywhere else for a repeat degree.

Mohammad Nazrul Islam
June, 2024

Certification

This is to certify that the dissertation titled **"Investigation on Fowl Adenovirus (Group 1) Circulating in Bangladesh and Molecular characterization of Specific Serotypes Associated with Inclusion Body Hepatitis"** prepared by the examinee bearing the registration No.: 1205017, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, to be awarded the degree of Doctor of Philosophy (PhD) in Microbiology.

To the best of my knowledge, the work is original and unique, and no component of the dissertation has been produced anywhere for any degree.

Professor Dr. Md. Mostafizer Rahman
Supervisor
Department of Microbiology,
Hajee Mohammad Danesh Science and Technology University,
Dinajpur- 5200
Bangladesh

Acknowledgments

I want to express the deepest sense of gratitude, all sorts of praises to the Almighty Allah, the omnipotent, omnipresent, and omniscient being whose blessing has enabled me to complete my thesis. A journey is made simpler when we go together; I have been accompanied and supported by a large number of individuals. It is a pleasant aspect that I now have the opportunity to thank them all.

The author would like to express his heartfelt gratitude and profound regards to his reverend teacher and research supervisor, Dr. Md. Mostafizer Rahman, Professor, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, for his scholastic and dynamic guidance, constant inspiration in every stage of this study, constructive criticism and encouragement throughout the period of research and manuscript preparation.

The author would also like to express his heartfelt appreciation to his respected co-supervisor, Dr. Md. Khalesur Rahman, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, for his kind cooperation, inspiration, valuable advice, and nice comments throughout the research work, which enabled the author to successfully complete the thesis.

The author also very grateful to Dr. Jahangir Alam, Chief Scientific Officer, Animal Health division, National Institute of Biotechnology, Savar, Dhaka, Bangladesh, for molecular analysis, sequencing, and phylogenetic tree analysis of the identified Fowl adenovirus.

The author finds a great pleasure in expressing his cordial thanks to honorable teachers, Prof. Dr. Md. Khaled Hossain, Prof. Dr. Mir Rowshan Akter, Prof. Dr. Md. Fakruzzaman, Dr. Atiqul Haque, Dr. Nazmi Ara Rumi, Dr. Farzana Afroz and Dr. Mst. Deloara Begum, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for

their valuable advice and constant inspiration throughout the entire period of the study.

The author is also grateful to Dr. Mizanur Rahman, CEO, AR AGROVET LAB, for his kind assistance, as well as to the commercial broiler, breeder, and commercial layer farmers and farm attendants for their cooperation during sample collection and reporting of farm situation and disease condition.

It is a limitless chance for the author to express heartfelt gratitude to the Institute of Research and Training (IRT), HSTU, for partially funding this study.

The author would like to convey his heartfelt gratitude to the laboratory technicians and administrative personnel of Hajee Mohammad Danesh Science and Technology University (HSTU).

Last but not least, the author is eternally grateful to his cherished parents, Idris Miah and Asea Begum, and beloved wife Ferida Begum, as well as his dearest son Talha, daughter Nazifa, and Sumaiya, for their heartfelt blessing, sacrifice, and encouragement during his academic career.

Finally, the author wishes to express his heartfelt gratitude to all of his friends, colleagues, relatives, and well-wishers.

Mohammad Nazrul Islam

June 2024

Published Article

Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2023, Article ID 7253433, 11 pages
https://doi.org/10.1155/2023/7253433

Research Article

First Evidence of Fowl Adenovirus Induced Inclusion Body Hepatitis in Chicken in Bangladesh

Mohammad Nazrul Islam,[1] **Md. Mostafizer Rahman**,[1] **Md. Khalesur Rahman**,[1] **and Jahangir Alam**[2]

[1]*Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Basherhat, Dinajpur, Bangladesh*
[2]*Animal Biotechnology Division, National Institute of Biotechnology, Ganakbari, Ashulia, Savar, Dhaka, Bangladesh*

Correspondence should be addressed to Jahangir Alam; alamjahan2003@yahoo.com

Received 23 September 2022; Revised 28 November 2022; Accepted 20 December 2022; Published 3 January 2023

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LISTS OF ABBREVIATIONS AND SYMBOLS

µL	Microlitre
AAV	Avian adenovirus virus
Ab	Antibody
AGID	Agar gel immunodiffusion
APC	Antigen-presenting cells
CAV	Chicken Anemia Virus
CD	Cluster of determinant
CPE	Cytopathic effect
CELO	Chicken embryo lethal orphan virus
CMI	Cell Mediated Immunity
CV	Coefficient of variation
DAI	DNA dependent INF-regulatory factor
DC	Dendritic cell
DNA	Deoxyribo Nucleic Acid
dph	Days post-hatch
dpi	Days post-infection
dpv	Days post-vaccination
ds	Double stranded
ds RNA	Double Stranded Ribo Nucleic Acid
ECE	Embryonated Chickens Eggs
ELISA	Enzyme Linked Immunosorbent Assay
EDS	Egg drop syndrome
FAdV	Fowl adenovirus
FC	Fowl Cholera
GDP	Gross Domestic Product
GE	Gizzard Erosion
HadV	Human adenovirus
HE	Hemorrhagic Enteritis
HHS	Hepatitis-hydropericardium syndrome
HRM	High resolution melt curve
IBDV	Infectious Bursal Disease Virus

IBH	Inclusion body hepatitis
IBV	Infectious Bronchitis Virus
ICTV	International Committee on Taxonomy of Viruses
Ig	Immunoglobulins
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INIB	Intranuclear inclusion bodies
IL	Interleukin
INF	Interferon
mAb	Maternal virus-neutralizing Antibody
MDA	Maternally Derived Antibody
MDV	Marek´s Disease Virus
MHC	Major histocompatibility complex
MSD	Marble spleen disease
MtAb	Maternal antibodies
NDV	Newcastle disease virus
NK	Natural killer
NLR	NOD-like receptors
NOD	Nuclear oligomerization domain
OD	Optical density
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNPP	p-Nitrophenyl Phosphate
PRRs	Pattern recognition receptors
QB	Quail Bronchitis
RFLP	Restriction fragment length polymophism
RIG-I	Retinoic acid inducible gene
RNA	Ribo Nucleic Acid
rt- PCR	Real-time polymerase chain reaction
S/P	Sample to Positive
SPF	Specific Pathogen Free

SPSS	Statistical Package for Social Science
ss	Single stranded
Th	T helper
THE	Turkey Hemorrhagic Enteritis
TLR	Toll-like receptors
VNT	Virus neutralization test
W/v	Weight by volume
Min	Minutes
Sec	Seconds
°C	Celsius

Abstract

Fowl adenoviruses (FAdVs) cause numerous diseases resulting in economic losses to the poultry industry worldwide. Several FAdV serotypes cause inclusion body hepatitis in chicken. Although FAdV infection was suspected, there was no confirmatory report from Bangladesh. The study was conducted to investigate the FAdV infection and specific antibodies in chicken. A total of 50 samples, each composed of liver and spleen, were collected from different chickens of Gazipur, Dinajpur and Panchagarh district. Three suspected organ samples derived from liver were also inoculated in to SPF embryonated chicken eggs for viral propagation. Each location is belonging to A, B and C poultry zones of Bangladesh, respectively. Viruses were detected by real-time PCR and conventional PCR. Blood samples (*n=303*) were collected at beginning and after recovery from infection and tested by indirect ELISA. Sequencing of PCR products were done for phylogenetic analysis. The indirect ELISA serological technique was employed which detects antibodies to Group-1 FAdVs and does not differentiate specific serotypes. Specific serotypes were detected through real time PCR and sequencing data analysis. All the flocks exhibited clinical symptoms of anorexia, drowsiness, ruffled feathers, reduced body weight, lack of uniformity and high mortality. Enlarged friable liver with yellow to tan color mottled with focal soft area, fluid in pericardial sac, swollen and hemorrhagic kidneys, enlarged congested spleen and hemorrhagic pancreas were found on postmortem examination. FAdVs were detected in 9 flocks (90%) out of 10 flocks except commercial layer flock from Dinajpur. Three serotypes namely serotype 8b in 7flocks (70%), serotype 11 in 2 flocks (20%) and serotype 5 in 1 flock (10%) were detected. The antibody titer increases significantly ($p<0.05$) after recovery from the infection. Phylogenetic analysis revealed that the Bangladeshi FAdVs have close identity with viruses from Asia, Europe, South and North America. These results implied that FAdVs have been introduced to Bangladesh multiple times. In this situation vaccination against detected particular serotypes associated with IBH (Inclusion Body Hepatitis) and upholding comprehensive biosecurity measures are crucial for the prevention and control of the disease.

Keywords: Fowl Adenovirus, Inclusion body hepatitis, Polymerase Chain Reaction, Indirect ELISA, Serotypes, Molecular detection, Bangladesh.

CHAPTER-1

INTRODUCTION

Bangladesh is predominantly an agricultural country. Livestock is an integral part of the complex farming system in Bangladesh as it not only a source of meat protein but also a major source of farm power services as well as employment. The livestock sub-sector provides full time employment for 20% of the total population and part-time employment for another 50% (Rahman *et al.,* 2014). Poultry is a rapidly growing sector of livestock and its expansion is being driven by rising incomes and shift in industry structure. The chicken population is about 30.41 million in Bangladesh and poultry farms are growing at a rate of 15% a year and the poultry meat alone contributes a substantial 37% of the total meat production in Bangladesh (Begum *et al.,* 2011). At constant prices, the contribution of livestock sector to the GDP in FY 2021-22 was 1.90 percent and the contribution of livestock to the overall agricultural sector was 16.52 percent (BER, 2022). The rural economy is revolving around livestock animals. Broiler and layer farming are important in supporting the livelihoods of poor, small, medium scale entrepreneurs, farmers, traders and consumers throughout the developing world including Bangladesh. The greatest impact of poultry in sustainable development designed to help the poor is enhancement of livestock-production systems (Dar *et al.,* 2015). However, the poultry farmers including broiler farmers still facing several challenges including disease outbreak causing a huge loss in poultry production due to high morbidity and mortality (Saleque, 2020). Every year several diseases like Newcastle disease (ND), infectious bursal disease (IBD), infectious bronchitis (IB), egg drop syndrome (EDS), fowl cholera (FC) broken out throughout the country resulting huge economic losses (Rahman *et al.,* 2019).

FAdVs causes a variety of diseases in chickens including inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS) and gizzard erosion and ulceration. FAdVs serotype FAdV-4 of species C is the causative agent of HHS and serotype FAdV-1 of species A has been isolated from most

cases of gizzard erosion and ulceration (Niu *et al.,* 2018, Gomis *et al.,* 2006). IBH is an acute disease caused by FAdV-D serotype 2, 3, 9, 11 and FAdV-E serotype 6, 7, 8a, 8b (Gulhane *et al.,* 2016, Schachner *et al.,* 2018).

The virus is transmitted by vertically as well as horizontally (McFerran *et al.,* 2003) has a worldwide distribution (Li *et al.,* 2017) and causes infection mainly in broiler chickens of 3-7 weeks of age, resulting sudden increase in mortality, which occasionally might be as high as 30% (Hess, 2013). IBH outbreaks have been confirmed in different countries and serotypes 2, 4, 8a, 8b and 11 have been reported as the most frequent serotypes as a causal agents (Wibowo *et al.,* 2019, Nakamura *et al.,* 2011, Choi *et al.,* 2012). The pathogenicity of recently isolated strains, mainly of FAdV serotypes 8b and 11 have been investigated with inconsistent results. Some studies had shown less to severe clinical signs and moralities when infecting chickens of different ages, like from 1 day to 3 weeks of age, with FAdV serotypes 8b and 11 (Dutta *et al.,* 2017, Zhao *et al.,* 2015). Phylogenetic analyses of partial hexon gene sequences are an adequate and quick method for differentiation and genotyping (Morshed *et al.,* 2017). Natural outbreaks of IBH are characterized by a sudden onset of mortality which peaks within 3-4 days and return to normal by days 5-6. Mortality usually ranges from 5% to 10% but can reach 30% (Hess, 2013, Schachner *et al.,* 2016). The morbidity is low and sick chickens adopt a crouching position with ruffled feathers (McFerran and Smyth, 2000). Polymerase chain reaction (PCR) with primer sequences based on the *hexon* gene or the *52K* gene is useful for virus identification (Mohamed *et al.,* 2018, Raue, *et al.,* 1998, Ahmed *et al.,* 2020, Gunes, *et al.,* 2012).

Besides, PCR together with DNA sequencing and/or restriction enzyme analysis has been used for FAdVs typing (Cizmecigil *et al.,* 2020). Real-time PCR and subsequent high resolution melting (HRM) curve analysis of three regions of the hexon gene were also reported and assessed for their potential in differentiating 12 FAdVs reference serotypes (Steer *et al.,* 2009). Also a SYBR

Green based real-time polymerase chain reaction is reported for detection and quantification of all FAdVs (Gune *et al.,* 2012).

I have been working directly in the poultry industry since 2005 (around 20 years). Based on my experience, incidence and disease history, FAdV infection in broiler parents was reported from Bangladesh in 2002 on the basis of a serological test named agar gel diffusion test (Biswas *et al.,* 2002). During last few years, clinical sings like sudden high mortality at very early age, lower growth rate and higher feed conversion ratio in commercial broiler and breeders of Bangladesh have been experienced by farmers and veterinary practitioners and suspected the causative agent may be FAdV virus (Saleque, 2020), also mentioned that antibiotic treatment was not responded and none of the suspected IBH cases were confirmed by laboratory test. However, no one reported the incidence of this disease based on molecular characterization of FAdVs to find out the specific causal agents and their serotypes. On the other hand, due to limited cross protection among 12 Fadv serotypes, circulating specific serotypes must be detected before using IBH vaccines. Because of this, I was very interested to investigate the specific causal agents of the disease and to find out preventative measures. In this study we have clinically, serologically and virologically investigated and reported the FAdVs in suspected chickens from three geographically different locations of Bangladesh. So far, this is the first report showed the molecular detection of virus along with sequencing and phylogenetic analysis of circulating serotypes as well as serological evidences of FAdV infection in Bangladesh. Therefore, the present study undertaken with the following specific objectives:

1. Clinical and serological investigation on fowl adenovirus (group 1) circulating in Bangladesh

2. Molecular characterization and phylogenetic analysis of specific serotypes associated with Inclusion Body Hepatitis.

CHAPTER-2

REVIEW OF LITERATURE'S

2.1 History

The word "adenovirus" was initially used to describe a virus that was discovered in human adenoid tissue cell cultures. The word was derived from the Greek "aden," which means gland (Rowe *et al.,* 1953). Earlier, the disease had been described, and its probable aetiology as a virus was proposed (Cowdry and Scott, 1930). Later on, the adenovirus of infectious canine hepatitis was identified (Cabasso *et al.,* 1954). The first avian adenovirus (AAV) was identified from chicken embryos infected with calf tissue during research on the aetiology of lumpy skin disease (Van den Ende *et al.,* 1949). Olson (1950) isolated the QB (quail bronchitis) virus from West Virginia quail suffering from an acute respiratory disease with high mortality. Yates *et al.* (1957) identified a novel virus and termed CELO (chicken embryo lethal orphan) virus which was first discovered in viable chicken embryo. Adenoviral research was sparked in the 1960s by the frequent isolation of CELO virus from eggs and egg-based vaccinations and its widespread seropositivity in chicken flocks (Oxford *et al.,* 1969). The earliest hepatic changes in broiler flocks at 5 weeks of age were described by Helmboldt and Frazier (1963). Stellate hemorrhages and circular margins on the enlarged livers, 90% of the parenchyma displayed fatty transformation and cowdry Type A inclusion bodies, according to the description of the histological abnormalities, which was called an “acute hepatic catastrophe”. Comparable pathology results and varying mortality in broiler chickens in succeeding years found in Canadian research. The livers were resembled enlarged and speckled with a reticulated pattern (Petits *et al.,* 1972**)**. Then the etiological agent was isolated and the first official nomenclature of FadV was mentioned (Bickford *et al.,* 1973). The condition known as MSD (marble spleen disease) caused by Fadv was discovered in spleens of ring-necked pheasants in 1966 (Mandelli *et al.,* 1966). Hemorrhagic enteritis (HE) in turkey was discovered to be caused by an

adenovirus in the mid-1970s (Pomeroy *et al.,* 1937). EDS (Egg drop syndrome) outbreaks were initially observed in laying hens with soft shells and shell-less eggs in the Netherlands in 1976 (Van Eck *et al.,* 1976). The Aviadenovirus was identified in broiler breeder hens with enlarged spleens and pulmonary edema approximately 3 years later (Domermuth *et al.,* 1979), but the disease was sporadic and as a subclinical infection. The first report of sudden and severe mortality in broiler hens aged 3-6 weeks by Fadv came from Pakistan in 1987 along with hepatitis hydro-pericardium syndrome named Angara Disease (Khawaja *et al.,* 1988). IBH and HHS outbreaks caused by several FAdV serotypes have been documented worldwide in recent years. Fadv serotypes 2, 8a, 8b, 11, and 4 are most often involved, and the majority of these outbreaks have been linked to genotypes FAdV-D, -E, or -C (Chen *et al.,* 2019).

2.2 The etiological agent

2.2.1 Taxonomy of the Adenoviridae

A variety of vertebrates, such as fish, birds, amphibians, reptiles, and mammals, are susceptible to adenoviruses (Harrach *et al.,* 2011). The Adenoviridae family was classified into two primary genera, according to Benko and Harrach (1998): Mastadenovirus and Aviadenovirus. These genera included viruses that invaded mammals and birds, respectively. Avian adenoviruses have been further classified into groups I, II, and III. Groups II and III of chicken adenoviruses contained unconventional poultry adenoviruses that differed from group I serologically (McFerran and Smyth, 2000). Adenoviruses belongs to group 1 are members of the genus Aviadenovirus of Adenoviridae family. Group I consist of 12 FAdV serotypes isolated from various avian species and sharing a common set of antigens and five different species, namely FAdV-A to FAdV-E based on their molecular structure (Toro *et al.,* 2000). Twelve groups of serotypes, such as FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11, have been identified based on the findings of cross-neutralization testing. Serotypes FAdV-1 and FAdV-5 are found in species FAdV-A and FAdV-B, respectively; serotypes FAdV-4 and FAdV-10 are

found in species FAdV-C; serotypes FAdV-2, FAdV-3, FAdV-9, and FAdV-11 are found in species FAdV-D; and serotypes FAdV-6, FAdV-7, FAdV-8a, and FAdV-8b are found in species FAdV-E (Fauquet *et al.,* 2005). The family Adenoviridae was reclassified in 2011 by the ICTV (International Committee on Taxonomy of Viruses) into five genera: Mastadenovirus, Atadenovirus, Siadenovirus, Icthadenovirus, and Aviadenovirus in their ninth report, based on the molecular criteria evaluation (Besson *et al.*, 2020).

2.2.3 Viral Structure

The FAdVs are non-enveloped and possess linear 35-36 kbp double-stranded DNA with icosahedral morphology and a diameter of 70-90nm, *as* determined by electron microscopy (Nicklin *et al.,* 2005). The viral DNA contains 53-59% guanine/cytosine. The viral genome contains 40 proteins, and the terminal segments of genomes are linked to related proteins. Capsid proteins include hexon, knobbed fiber, and penton, while cement proteins and core proteins are the other minor structural proteins (Russell, 2009). The viral capsid is made up of 720 hexon subunits arranged as 240 trimers and 12 triangular penton capsomers with one or two protruding strands (Viralzone, 2015).

The interaction between penton and fiber with the receptors of infected cells has been demonstrated (Jucker *et al.*, 1996; Fingerut *et al.,* 2003). The hexon gene varies according to virus serotype, with the largest one being 967 amino acids. H1, H2, H3, and H4 are the four types of hexons (Burnett., 1985). H1 hexons have 12 apices of connection to pentons, making them peri-pentonal. Across the 20 faces of the icosahedra, there is a cluster of nine additional hexons.. On the other hand, H2 and H3 are the twofold and threefold axes, respectively. The remaining ones are H4, correspondingly. At the apex of the hexon molecule, there are nine hyper variable areas. Because of the presence of these hyper-variable areas, hexons are very sensitive to mutations, they contain the primary neutralizing epitope, these hexons could be utilized for serotyping (Rux *et al.,* 2003; Roberts *et al.,* 2006 and Matsushima *et al.,* 2011). Cell surface binding receptors and pathogenicity epitopes are found in the fiber.

Thus, via interacting with cellular receptors, fiber protein is responsible for viral capsid attachment to the host cell surface (Russell., 2009). With separate receptors, FAdVs have one long fiber and two short fibers. One fiber is in charge of virus attachment, while the others are in charge of virus internalization (Tan *et al.,* 2001). The knob, shaft, and tail make up the fiber. The fiber knob influences the haemagglutinating properties of virus, which are utilized to classify species (A-E) (Pehler- Harrington *et al.,* 2004). Furthermore, the knob is involved in the synthesis of fiber protein and encapsidation (Henning *et al.,* 2006). The fiber contains around 582 amino acids that bind to the penton base. The penton is made up of base and fiber protein. It has been demonstrated that the penton base affected by heat, trypsin, pH, and ionic strength variations (Zubieta *et al.,* 2005). A neutralizing antibodies against the penton is produced in blood sera & the penton plays a significant role in the penetration of virus into the host cell (Hong *et al.,* 2003). Non-structural proteins with the numbers 100K and 33K are important. During the reproduction of viruses of groups B and C in insects, the 100K protein aids in intracellular transport and hexon folding (Hong *et al.,* 2005). In order to differentiate vaccinated from unvaccinated infected birds with FadVs, antibodies against these proteins were used (Xie *et al.,* 2013).

2.3 Epidemiology

The Poultry of all ages and species, including falcons, raptors, ostriches, psittacines, and parrots, are susceptible to infection by avian adenoviruses, which are members of the adenoviridae family (Hess, 2020). Fowl adenoviruses infection was reported in Australia (Reece *et al.,* 1986), in New Zealand (Christensen *et al.,* 1989), in Asia & the Middle East (Xia *et al.,* 2017), in Europe (Oliver-Ferrando *et al.,* 2011-2013), in Africa (Joubert *et al.,* 2014) in the past ten years. The incidence of these viruses has been increased significantly in these countries. The diversity of fowl adenoviruses is demonstrated by the fact that they have been found to infect at least 40 distinct species of vertebrates, including fish, mammals, and birds (Benko and Harrach,

2003; KoG, 2005). Birds with and without diseases have been found to harbor FAdV infections (Niczyporuk *et al.,* 2012). It is evident that as host age increases, both the degree of FAdV multiplication and the losses diminish (Rahimi and Minoosh, 2015). Several bird species, including chickens, turkeys, ducks, and geese, have been infected by fowl adenoviruses (Pan *et al.,* 2017a). Approximately 31 species of birds have been documented to play a role in the spread of FAdV outbreaks (Hess., 2000**)**.The susceptibility to FAdVs infections also has been documented in wild black kites (Kumar *et al.,* 2010), guinea fowl (Zellen *et al.,* 1989), falcons (Singh *et al.,* 2002) pigeons (Steer *et al.,* 2009) and parrots besides commercial poultry (Bradley *et al.,* 1994).

2.4 Transmission

2.4.1 Horizontal transmission

In poultry, horizontal transmission is the most frequent mechanism of FAdV transmission. Infected farm-workers, vehicles, and equipment can transfer the virus to flocks; aerosols are rarely the means of transmission. Birds that are sub-clinically or clinically sick release FAdV into the environment through their feces or respiratory secretions (Adair *et al.,* 2008). Susceptible birds are also infected orally. Fecal shedding of FAdV varies by species and age, and it is influenced by the immunological status of birds. Adult birds only temporarily excrete a low titer of the virus in their feces, but young birds periodically shed FAdVs for prolonged periods of time. Moreover, peak shedding periods vary significantly between different species of FadVs (Clemmer, 1972). The development of virus-specific cellular and humoral immunity will cause to cease fecal shedding of viruses (Dawson *et al.,* 1979).

2.4.2 Vertical transmission

In addition to horizontal transmission, FAdVs are transmitted from broiler breeder parents to their broiler progenies vertically (Mazaheri *et al.,* 2003). In order to understand how the CELO virus and other FadVs spread through eggs, laboratory researches are conducted and field examinations of FAdV infections

are observed. In broiler chickens, vertical transmission of FadV-1 and FadV-4 has been documented experimentally. These serotypes have been resulted in gizzard erosions and ulcerations and HHS respectively. FAdV also may be caused latent infections in chickens and reactivated at sexual maturity. FAdV-8 viral antigens have been discovered in egg contents and in tissues of chickens during some field investigations (Grafl *et al.,* 2012; Reece *et al.,* 1987). The timing of the vertical transmission is another issue that has to be investigated . Vertical transmission of the FAdV-1 and CELO viruses takes place up to seven days after infection (Grgic *et al.,* 2006). In addition to the duration of transmission, researchers have connected vertical transmission to the development of specific antibodies. According to one group of researchers, seroconversion of experimentally infected chickens caused vertical transmission to cease (Dawson *et al.,* 1981). However, when a heterologous virus is isolated from broiler chickens that came from vaccinated parent flocks against different serotype, or when the disease manifests earlier than its typical course, which occurs between two and five weeks of age, vertical transmission is often expected (Pilkington *et al.,* 1997; Senties-Cue *et al.,* 2010).

2.5 Pathogenosis

The first replication of FadV viruses occurs in the large and small intestines once they enter the host. Then the spread of viruses into several organs, including the liver, kidney, respiratory system, bone marrow, and bursa, results in viraemia. It is possible to isolate viruses from the bursa of Fabricus, nasal mucosa, ocular mucous membranes, and feces. Once infected, the chicken becomes a lifetime carrier of the adenovirus; viruses may also spread through eggs (Anjum,;1990). The three stages of the infection process are incubation (1-3 days post-infection), degeneration (4–7 days post-infection), and convalescent (12 days post-infection) based on the development of pathological lesions have been observed (Steer *et al.*, 2015). The aberrant metabolic markers such hypoglycemia and elevated pancreatic lipase activity in the sera of affected birds are found during the acute phase when hepatic and pancreatic

lesions predominated (Matos *et al.,* 2016). When viral replication is completed, the virus causes cellular lysis and exits the body by respiratory or fecal excretion. If chickens are producing eggs, the virus may be transmitted to the eggs and depending on the immunological condition of birds, it is possible that there will be mortality in offsprings (Adair *et al.,* 2008). In experimental studies, the lethality of FAdVs is dependent on several parameters, including as the strain or serotype of the virus, the age, line, immune status of the birds, and the mode of infection (Matos *et al.*, 2016).

2.6 Diseases caused by fowl adenovirus

2.6.1 Quail bronchitis

Acute, catastrophic, and highly contagious pulmonary quail bronchitis causes considerable economic losses in juvenile bobwhite quails (Barnes, 1987). Similar to the QB virus in terms of serological characteristics, lesions, and pattern of death the Chicken Embryo Lethal Orphan Virus (CELOV) is isolated from chickens (DuBose and Grumbles, 1959). The statement suggests that QBV and CELOV are considered to be belonging to the same strain type in relation to FAdV group I and serotype 1 (Cowen and Calnek, 1975). After experimental inoculation in quails, both viruses might cause bronchitis. However, it has not been demonstrated that either QBV or CELOV may infect non-quail species (DuBose *et al.,* 1958). The QBV was isolated from five to eight-week-old bobwhite quails in Minnesota, USA, and its molecular identification was determined. It was a member of the FadVs-1 (species A). This instances demonstrated visible lesions, respiratory symptoms, and mortalities. Ninety-nine percent of the nucleotide sequences of the FAdV isolates analyzed in this investigation were identical to the FADV group-1 CELO strain. FAdV species A and QBV isolates were closely related, whereas FAdV species B–E were different (Singh *et al.*, 2016).

2.6.2 Inclusion body Hepatitis

The disease IBH (Inclusion body Hepatitis) was identified in poultry of the United States in 1963 (Helmboldt and Frazier, 1963). Poultry has been affected by inclusion body hepatitis as a costly and devastating disease all over the world (Schachner *et al.,* 2016; Schachner *et al.,* 2018). It has been introduced to Australia, Europe, Canada, India, Turkey, Saudi Arabia, and Egypt rapidly. Sometimes an IBH infection lasts for two to three weeks.The mortality rate of IBH in broilers up to five weeks old ranges from zero to 5- 10% and can reach 30% over a brief period (average five days) of time (Alvarado *et al.,* 2007). Extremely high death rates (60–70%) due to IBH have been observed in Canada and India. Variable moralities may be related to the pathogenicity of virus strains, age, susceptibility and the concomitant immunosuppressive diseases (Dahiya *et al.,* 2002). In broiler and layers enlarged, pale, necrotic livers, as well as petechial and ecchymotic skeletal muscle hemorrhage were found in postmortem examination, in falcons splenomegaly and lymphoid atrophy in the bursa of Fabricius were seen (Ahamad *et al.,* 2016; Schrenzel *et al.,* 2005). Histopathological investigations of the IBH infected liver revealed varying areas of multi-focal hepatocellular necrosis, lymphoid infiltration and vascular degeneration (Wilson *et al.,* 2010). Degenerated hepatocytes had large, spherical, or irregularly shaped intranuclear basophilic inclusion bodies; these inclusions were typically visible six to nine days following infection (Grimes *et al.,* 1977; Steer *et al.,* 2015). The liver, pancreas, and spleen have all been shown to have inclusion bodies, which indicates that adenovirus replication takes place in these organs (Cook, 1983). At first, FAdVs-8 (group I) was identified as the particular serotype of IBH (Erny *et al.,* 1991). It has been suggested by many research that FAdVs-2, -3, -9, and -11 (D) are the main causes of IBH (Ojkic *et al.,* 2008). Epidemiological studies on IBH outbreaks in Canada have identified FAdVs-2, -6, -7, -8 a, b, and -11 in broiler flocks (Ojkic *et al.,* 2008). FAdVs-8b and FAdVs-7 in broiler chickens were found to be the causes of IBH in Slovenia and Poland, respectively (Zadravec *et al.,* 2013 ; Niczyporuk, 2017). There have been reports of IBH epidemics in

China (Zhao *et al.,* 2015), Korea (Choi *et al.,* 2012), and New Zealand (Christensen and Saifuddin, 1989) that are caused by FAdVs-2, -4, -8a, b, and -11. Australia, Austria, Spain, and South Africa have reported IBH outbreaks linked to FAdVs-8b or -11 (Maartens *et al.,* 2014; Schachner *et al.,* 2016; Oliver-Ferrando *et al.,* 2017). The virus was discovered in 2012 after an epidemic at a 21-day-old broiler chicken farm in Iran, which resulted in 14% mortality (Rahimi and Minoosh, 2015). The IBH outbreaks in Iran from 2013 to 2016 were associated with FadVs-11 (species D) and -8b (species E) (Hosseini and Morshed, 2012; Nateghi *et al.,* 2014). FAdVs-8a , FAdVs-2, and FAdVs--11 from chickens were detected in Egyptian broiler flocks (Radwan *et al.,* 2019). In Saudi Arabian falcons and broilers, FAdVs-2 and FAdVs- -6 were shown to be the causal agents of IBH (Mohammed et al. 2018). There is a closer genetic relationship between FAdV species D and E (Marek *et al.,* 2013). It has been suggested that immunosuppressive diseases such MD, CAV and IBD contribute to the spread of IBH and increasing death rate (Markowski-Grimsrud and Schat, 2003, Fadly *et al.,* 1976). But in the absence of additional immunosuppressive factors, it has been shown that IBH also can result in fatalities (Ojkic *et al.,* 2008).

2.6.3 HHS (Hepatitis Hydropericardium Syndrome) /Angara disease

In 1987, an outbreak of HHS/Angara disease was identified in Pakistan, affecting broilers between three and five weeks of age (Jaffery *et al.,* 1988). With a 20–80% death rate, this disease has now spread throughout the world. It also causes nephritis, pulmonary edema, and an enlarged, friable, hemorrhagic liver with INIB, all of which are brought on by the pathogenic FadV serotype-4 virus, which is a member of the FadV species C (Li L *et al.,* 2016). The presence of a characteristic hydropericardium is the primary distinction between the symptoms of IBH and HHS. Because the hydropericardium surrounding the hearts of affected birds resembled the Indian lychee fruit, HHS was known as "Lychee heart disease" (Gowda *et al.,* 1994). FAdVs-4 (C) is the primary cause of HHS, according to multiple research (Nakamura *et al.,*

2000; Asthana *et al.,* 2013). Three to six week old broilers are susceptible to the poultry adenovirus disease known as HHS. The illness is characterized by high death, lung congestion, nephritis, hydropericardium, and urate deposits in the kidneys (Balamurugan *et al*., 2004). Hemorrhages on the heart muscles and other organs, as well as straw-colored, watery, or jelly-like fluid in the pericardial sac, are indicative of gross lesions of HHS. Additionally, reports of bursa swelling, edema, pale kidneys, enlarged, pale, and friable liver, congestion in the lungs, and so on have been reported (Ganesh and Raghavan, 2000; Ahmad *et al.,* 2011). Hydropericardium syndrome is a highly pathogenic infectious disease with a low morbidity rate that mostly affects young broiler chickens (Akhtar, 1994). The mortality rates of broilers may range from 20% to 75% (Cheema *et al.,* 1989), 30-80% (Ahmad *et al.,* 1989), or 30-60% (Zhao *et al.,* 2015). The disease occurs in commercial broilers about the third week of life and lasts four to eight days. Adult broiler breeders were occasionally afflicted with moralities reaching up to 6.4% (Abe *et al.,* 1998). The FAdV causing HHS was identified as serotype 4 by a serum-neutralization test, PCR and restriction enzyme analysis. Inoculation of isolated FAdV-4 subcutaneously and orally in 28-day-old broilers caused infection experimentally (Shukla *et al.,* 1997). The relationship of adenoviruses (serotype 4) isolated from India and Pakistan was verified by sequence analysis of the variable region hexon gene, which showed 94–98% similarity (Mansoor *et al.,* 2009). Recently, FAdVs-4 was extracted and molecularly characterized from the oviduct of layer chicken flocks in Eastern Japan that produced fewer eggs (Del Valle *et al.*, 2020).

2.6.4 Gizzard Erosion & Ulceration

Gizzard erosion was discovered for the first time in 1993. The natural host of GE (Gizzard erosion) is broiler and layer chickens (Tanimura *et al.,* 1993). GE was also found in bobwhite quails in North America (Goodwin, 1993). FAdVs-1 (species A) and FAdVs-8 (species E) are the most frequent causes of GE in Japan, England, Italy, Germany, Korea, Poland, and Iran; however, GE has also

been associated with experimental infection with other serotypes, including FAdVs-4, 8b, and 11. Following experimental oral and ocular inoculations of the FAdV-1 strain in one, three, and five-week-old broiler chickens, characteristic microscopic lesions have been found (Ono *et al.,* 2004; Manarolla *et al.,* 2009; Okuda *et al.,* 2004; Grafl *et al.,* 2015). The brown to black erosion zones in gizzards varied in size based on different disease conditions. Heterophils, lymphocytes, macrophages, plasma cells, and intranuclear basophilic inclusion bodies are observed along with multifocal or extensive cuticle koilin layer degeneration microscopically (Marek *et al.,* 2010). Following an experimental oral inoculation with FadV-1, one-week-old SPF hens in Korea showed significant glandular epithelial necrosis and degeneration together with eosinophilic inclusion bodies, as revealed by a histological study of the gizzard (Lim *et al.,* 2012). A post-mortem examination of 48 gizzards that were performed at seven commercial broiler farms in Iran revealed that koilin layer of the gizzards was perforated, roughened, and stained. Microscopic studies showed that desquamation of epithelial cells in the glandular mucosa, the mucosa, submucosa, and musculosa had mild to severe inflammatory cell infiltration, and cell debris at koilin surface (Mirzazadeh *et al.,* 2019). The outcomes that show the most consistency are the loss of homogeneity in broiler flocks and the condemnation of carcasses. Since chicken gizzards are highly desirable in Asian countries, rejecting them has significant implications for the economy (Adair *et al.,* 2008).

2.6.5 Hemorrhagic enteritis in Turkey

Hemorrhagic enteritis in Turkey was originally distinguished in the United States without the precise causal agent being identified in 1937. The researchers then hypothesized that the causal agent might be filtered using a 0.22μ filter, and the result demonstrated that the cause of Turkey Hemorrhagic Enteritis (THE) is a virus (Gale and Wyne, 1957; Gross and Moore, 1967). THEV is considered to be a virus that is a member of the Adenoviridae family and genus Siadenovirus. Adeno-like virus particles were discovered in the

spleen and intestines of infected turkeys. The results of molecular characterization of the hexon gene indicates that THEV is related to FAdV-3, a related strain of penguin adenovirus (Carlson *et al.,* 1974).

Turkeys that are six to eleven weeks old have a more severe case of the diseases. Due to the presence of maternal antibodies that protect turkeys during their early age of life, birds less than four weeks old are less susceptible (Fadly and Nazerian, 1984)). Turkeys with THE (Turkey Hemorrhagic Enteritis) are more likely to have transient immunosuppression, acute depression, and bloody diarrhea (Hoerr , 2010). Under subclinical conditions, the disease causes large economic losses because of its fast unexpected death (up to 80%), blood loss, anemia, and immunosuppression with subsequent bacterial or parasite infections (Chandra and Kumar, 1998.). Immunosuppression manifests itself by a reduction in immunological response to vaccines like New castle disease vaccines (Nagaraja *et al.,* 1985). Although turkey is the most common susceptable host, antibodies to THEV have been discovered in hens, quails, chukars and peafowl (McFerran and Smyth, 2000).

2.6.6 Egg drop Syndrome

A disease resulting in soft-shelled or shell less eggs and decreased egg production in a laying flock was first identified in the Netherlands (Van Eck *et al.,* 1976). The main source of haemagglutinating FadVs in Northern Ireland was laying hens (McFerran *et al.,* 1978). Eventually, the disease was identified as Egg Drop Syndrome (EDS) and seen in several countries around the globe (Firth *et al.,* 1981). There have been reports of antibodies against the EDS virus in chickens from Denmark, Brazil, Mexico, Nigeria, and New Zealand (Nawathe and Abegunde, 1980; Howell, 1982). Moreover, wild ducks and pigeons have been found to contain EDSV antibodies (Gulka *et al.,* 1984). The infection may spread to pheasants, guinea hens, and quail from infected chicken flocks. EDSV has been identified as duck adenovirus 1 belonging to Atadenovirus genus of the Adenoviridae family (Dán *et al.,* 1998). Despite the

fact that EDSV infections most frequently occur spontaneously in waterfowl, such as ducks and geese (Schlor, 1980). Turkeys are susceptible to experimental infection with ESDV, even though they do not show any symptoms (Parsons *et al.*, 1980). A 50% reduction in egg production that lasts four to ten weeks is a characteristic of EDS epidemics (Alam *et al.*, 2009). Three to seven weeks of a 20–25% decrease in egg production was recorded during the first EDS outbreak in around 40 week-old broiler breeder farm in Japan. Abnormal external egg quality (such as discoloration, soft shell, thin, or shell-less eggs) was typical in cases with EDS infection (Yamaguchi *et al.*, 1981). Sero-positive instances of EDSV were found in layer flocks in Bangladesh (Biswas *et al.*, 2009), leading to lower egg production and soft-shelled or shell-less eggs. Infected quail had a decrease in egg production, increased in the number of soft-shelled eggs, and the formation of EDS-inhibiting antibodies (Das and Pradhan, 1992). Respiratory symptoms in goslings due to EDSV has also been documented (Ivanics *et al.*, 2001). The eggshell gland of the oviduct is the primary site for virus multiplication and as a result, deformed eggs are produced (Smyth *et al.*, 1988).

2.7 Diagnosis of Aviadenoviruses

Clinical diagnosis based on particular signs and lesions is challenging and non-confirmatory. The presence of particular lesions as well as inclusion bodies under the microscope, molecular detection or serology may be diagnostic for FAdVs infections. After staining of tissue homogenates, electron microscopy is successfully employed to determine virus morphology (Chandra *et al.*, 1997).

2.7.1 Clinical findings & Gross lesions

There have been reports of sudden death in birds that were 4 days to 6 weeks old. The typical range of mortality rates was 40% to 60%. HHS mortality usually results in 20–80% of fatalities and starts at three weeks of age. The primary pathogenic characteristic of HHS is the development of a clear, straw-colored fluid in the pericardial sac. Localized hepatic necrosis, enlargement,

pale to tan color, and intranuclear inclusion bodies are characteristics of the classic form of IBH. It was found that the birds were huddled together, ruffled feathers, and their droppings were yellow. The kidneys appear pale, enlarged, and speckled (Cheema *et al.*, 1989).

2.7.2 Isolation of viruses

FadVs can be grown in embryoned eggs via the chorioallantoic and yolk-sac methods (Winterfield *et al.*, 1973) as well as in primary cell cultures produced from chicken embryo fibroblast (Zsak, 1984), chicken embryo kidney cells, and chicken embryo liver cells (Schachner *et al.*, 2014; Schonewille *et al.*, 2010). In addition to primary cell cultures, many researchers have used chicken-derived continuous cell lines for FadVs. These are the chicken hepatoma cell line and the leghorn male hepatocellular carcinoma cell line (Alexander *et al.*, 1998). Homogenates of FadVs infected chicken embryos from 11 or 19 days old were successfully inoculated into tissue culture lines (Chandra *et al.*, 2000). To induce cytopathic effects, these viruses may need to be adapted through serial passages on cell lines ((Roy *et al.*, 2001). Within five to six days, inoculated viruses cause positive responses, or regions of cytopathic effects. When inclusion bodies are present, the cytopathic effect shows up as cell surface detachment (Khawaja *et al.*, 1988).

2.7.3 Serological examinations

Serological tests range from the simple Agar Gel Immunodiffusion Assay (AGID) to the more advanced enzyme-linked immunosorbent assays (ELISA). Serological assays track the immune response to routine immunization or diseases. These tests are crucial parts of epidemiological research aimed at determining the frequency of FAdV infections in flocks. Serological techniques, like AGID, have been used in a number of investigations to assess FAdV status of chicken (Girshick, 1980). However, due to low detection limit AGID, false negative results might occur. Many researchers have used ELISA in addition to AGID in experimental studies to assess antibody responses to

FAdV infection or rule out the FAdV status of flocks (Philippe *et al.*, 2007). Because of group-specific antibodies can mask some FAdV serotypes, AGID and ELISA can detect antibody responses but cannot discriminate between different FAdV serotypes (Calnek *et al.*, 1982). Virus neutralization assays (VNT) detect anti-FAdV fiber antibodies unique to the virus. FAdV serotypes are distinguished using this technique (Adair *et al.,* 2008).Viral-specific antibodies have also been utilized in immunohistochemical approaches to identify viral replication sites in tissues (Saifuddin *et.al,* 1990). It has recently been shown that ELISA by utilizing antibodies made against the FAdV fiber (FAdV-1, -2, -4, -8a, -8b, and -11) the specific fowl adeno viruses may be distinguished (Feichtner *et al.*, 2018).

2.7.4 Molecular examination

FAdVs have been shown to be easily detected utilizing real-time PCR (polymerase Chain Reaction) rather than conventional PCR (Gunes *et al.*, 2012). The diagnosis of FAdV has been revolutionized by molecular technique. PCR has been used alone or in conjunction with restriction enzyme analysis to separate FAdVs into different genotypes based on electrophoretic mobility in agarose gel (Meulemans *et al.*, 2001). The RFLP (restriction fragment length polymorphism) technique to differentiate between avian adenoviruses gained widespread recognition (Zsak and Kisary's, 1984). PCR and DNA sequencing of the hexon gene are utilized to categorize FAdVs into genotypes more accurately and to create phylogenetic analysis showing evolutionary connections with recently discovered viruses (Lim *et al.*, 2011; Whittaker and Helenius, 1998). Gene sequencing and 52K gene detection have been used to identify FAdVs (Kajan *et al.*, 2011). FAdVs can be distinguished into various species and serotypes by replicating certain hexon gene segments and subsequently utilizing restriction enzyme digestion or nucleotide sequencing (Meulemans *et al.*, 2001). Additionally, serotype-specific neutralizing epitope of hexon gene provides the basis for FAdV serotyping (Niczyporuk, 2017). FAdVs were divided into five different species (A–E) by RFLP (Hess, 2000).

Fiber-gene sequencing in addition to FAdV hexon gene sequencing has been used to differentiate FAdV serotypes (Schachner *et al.*, 2016). By utilizing a real-time PCR apparatus and high resolution melting curve (HRM) analysis of the hexon gene, scientists have created quicker methods for accurately and confidently genotyping FAdVs in a matter of hours. Although experimental results showed a high degree of coherence between genotyping and serotypes based on HRM analysis of the hexon gene, it is occasionally necessary to use several tests to identify the virus (Steer *et al.*, 2011).

2.8 Immune response to FAdV

Immune responses to infectious agents are divided into two main categories: innate immunity which is innate and non-specific, and adaptive immunity which is specific to a particular pathogen and develops over time and helps to eliminate the pathogen from the host body (Tizzard, 1987).

2.8.1 Innate immune response

Within minutes to hours, innate immunity begins to recognize the organisms and restricting their spread. Many substances, such as enzymes (lysozymes), antimicrobial peptides, innate immune cells like neutrophils, eosinophils, macrophages, NK (natural killer) cells, and dendritic cells, and humoral components, can trigger an innate immune response. These include physiological and anatomical barriers, as well as complement systems (Zachary *et al.*, 2016). Numerous pattern recognition receptors (PRRs) on innate immunity cells are capable of identifying pathogen-specific molecular patterns (PAMPs). A group of extracellular and intracellular receptors known as the PRRs are activated by PAMPs, which in turn triggers a number of downstream pathways. Cellular activity triggers the release of cytokines and chemokines to draw in more inflammatory cells (macrophages, neutrophils), antigen-presenting cells (APCs), and ultimately activates the adaptive immune response. Antimicrobial compounds (enzymes and peptides) are secreted to destroy the pathogen. In animals, the innate immune system responds to viral

infections by detecting viral PAMPs via PRRs. Viral PAMPs mostly consist of viral proteins, viral DNA, single-stranded (ss) RNA, double-stranded (ds) RNA, and RNA with 5' phosphatase ends**.** The main targets of antiviral innate immune responses are toll-like receptors (TLR-2, TLR-3, TLR-7, and TLR-9), nuclear domain (NOD) like receptors (NLRs), retinoic-acid inducible gene-I (RIG-I) like receptors, and DNA dependent interferon (IFN) regulated factors (DAI) (Kanneganti *et al.*, 2007, Petrilli *et al.*, 2007).

2.8.2 Adaptive immunity

The adaptive immune response in chickens, like that of mammals, has two arms: cellular and humoral, and it responds to viral infections by producing cytotoxic T-cell responses and antibody responses (Okino *et al.*, 2013).

Cell-mediated immunity (CMI) is composed of CD4+ (helper T-cells) and CD8+ (cytotoxic T-cells) responses that are dominated by IFN- responses, resulting in enhanced activation of macrophages and NK-cells (Thomas et al.; 2006). The enhanced expression of cytokines such as IL-4 and IL-10, as well as antibody production (extracellular immunity) by plasma cells (differentiated B lymphocytes), dominates humoral immunity (Sharma, J.M., *et al.,* 2000). Unlike mammals, which have five types of antibodies, birds only have three: immunoglobulin IgM, IgY (mammalian homologue of IgG), and IgA. IgM is the first antibody produced in response to infection, and it is replaced by IgY as the immune response matures (Carlander *et al.,* 1999). These two are critical in the defense of interior organs against infections. IgA has a vital role in protecting mucosal surfaces from infections (Sheela *et al.,* 2003). The key capsid proteins of viruses are the primary targets of adaptive immunity. Adenoviral main capsid proteins (fiber, penton, and hexon) can elicit both cellular and antibody-mediated response. Among the three capsid proteins, hexon is the most abundant and hence triggers the greatest cellular or antibody response (Molinier-Frenkel *et al.,* 2002). The parents are hyperimmunized to

stimulate the humoral arm of adaptive immune system to protect their progeny against viral diseases by maternally derived antibodies (Gharaibeh, 2008).

2.8.3 Adenoviruses as vaccine vectors

Vectored vaccines have been popular in veterinary medicine to safeguard animal health in addition to human medicine. In the previous decade, the poultry vectored vaccine market experienced unprecedented expansion & several viral vectored vaccines that have been approved for use in the veterinary industry (Meeusen *et al.,* 2007). MDV vectored vaccination has the earliest history for the poultry vector vaccine sectors. Its genome has been employed in the replication of numerous chicken viruses, including IBDV (infectious bursal disease virus) (Darteil *et al.,* 1995), NDV (Newcastle disease virus), avian influenza virus and infectious laryngotracheitis virus (Gimeno *et al.,* 2015). A recent study also demonstrated the development of an MDV-vectored avian leucosis virus subgroup-J vaccine ((Liu *et al.,* 2016). A variety of animal adenovirus vectored vaccines already developed to protect animal health, including Canine Adenovirus-2 expressing rabies virus glycoprotein, HAdV-5 expressing avian influenza virus hemagglutination, Bovine Adenovirus-3 expressing herpes virus group-D antigens, and many other viruses (Hu *et al.,* 2006; Reddy *et al.,* 2000). FAdV-1, FAdV-8, and FAdV-10 are first-generation replication competent of FAdV vectors which utilized to generate FAdV vectored IBDV vaccines expressing the viral protein-2 gene. In animal studies, these vectored vaccines demonstrated protective effectiveness against IBDV (Sheppard *et al.,* 1995; Francois *et al.,* 2004). Due to its low host pathology, another FAdV serotype (FAdV-4) has recently been investigated for use as a vaccine vector (Grgic *et al.,* 2013). Because of their non-infectiousness in many human origin cell lines, they are a promising choice for making vectors for gene therapy in humans, in addition to vectored vaccine research for poultry infections. CELO virus has been successfully used to express human interleukin (IL) genes in embryos as well as for gene therapy for malignancies (Cherenova *et al.,* 2004; Shashkova *et al.,* 2005).

2.9 Prevention and Control of IBH

Strict Biosecurity and farm cleanliness are critical to prevent & control FadV infections and outbreak of diseases. Besides adequate biosecurity, effective immunization program is necessary in the prevention & control. FAdV infections are transmitted to chickens either horizontally or vertically. Although good biosecurity can reduce the horizontal spread of virus, vertical transmission of FAdVs is impossible to avoid without vaccination of broiler breeders. Consequently, immunization of broiler breeders to develop antibodies against FAdVs is more crucial to prevent vertical transmitted disease (Adair *et .al.* 2008). Furthermore, it allows maternal antibodies to be transferred to progenies, protecting them from FAdV infection during neonatal life (Alvarado *et al.,* 2007). When hens are exposed to heterologous viruses, the maternal antibody barrier is broken, resulting in diseases in broiler progenies (Grimes, 1977). As a result, a successful preventive strategy necessitates vaccines that provide specific protection against specific serotypes (field isolates). These include live vaccinations, autogenous inactivated vaccines, cell culture and egg origin virus inactivated vaccines, and subunit vaccines (Li *et al.,* 2017).

2.9.1 Live Vaccines

For mass vaccination, live virus vaccines are inexpensive, effective, and simple to administer. To prevent infectious viral diseases of poultry, such as NDV, ILT (infectious laryngotracheitis), and IB (infectious bronchitis) numerous live vaccines have been used (Collett, 2013). In Australia, a monovalent live FAdV (FadV-8b) vaccine is currently approved for use in broiler breeder immunization programs to prevent broilers from IBH caused by FAdV-8b . The vaccination technique includes oral exposure of an increasing percentage of birds many times in early ages (before production) until appropriate antibody levels are developed. Aside from IBH, live FAdV vaccines against HHS (FAdV-4) have been produced by attenuating live FAdV-4 strains in embryonated chicken eggs. In these trials, vaccinated chicks showed up to

97.7% to 100% protection after lethal virus exposure (Schonewille *et al.*, 2010, Mansoor *et al.,* 2011).

2.9.2 Inactivated Vaccines

In many countries both breeders and commercial broilers receive inactivated immunizations. Proper vaccination protects offspring of breeders from both field infections and clinical diseases. Unvaccinated birds sometimes die before 10 days old due to a lack of serotype-specific anti-adenovirus antibodies or irregular MDA transmission due to poor vaccination. Research in Chile demonstrated that vaccinating broiler breeders against both CIAV and FAdV-4 improved progeny protection against HHS compared to vaccination against either disease alone (Toro *et al.,,* 2000). In India, only inactivated oil emulsion vaccines are used to prevent HHS during probable epidemics (Gowthaman *et al.,,* 2012). In Egypt, breeder chickens have been vaccinated with an inactivated FAdV-C serotype 4 vaccine since late 2020. However, the cross-protection of vaccines against isolated FAdVs D (serotype 2 and 11), and E (serotype 8 a, b) is unknown and requires further research (data under investigation). In breeders, the inactivated vaccinations are made with mineral oil or aluminum hydroxide adjuvants and injected intramuscularly into the pectoral muscles. In some areas, FAdV vaccinations are also manufactured as inactivated polyvalent vaccines alongside other avian viruses such as NDV and egg drop syndrome (Shah *et al.,* 2017). The protection rate with inactivated FAdV-4 against HHS was from 80 to 100% (Schachner *et al.,* 2017), 90- 100% against IBH (FAdV-2) (Junnu *et al.,* 2015], and 98% against FAdV-8 and 92% against FAdV-11 (Alvarado *et al.,* 2007).

2.9.3 Subunit vaccinations

Subunit vaccines have been developed and evaluated by a number of researchers in an effort to prevent FAdV diseases such as hemorrhagic enteritis, Egg drop syndrome, and HHS. In broilers, the FAdV-4 penton subunit immunization offered 90% protection against HHS. Fiber-1 and fiber-

2 FAdV-4 subunit-vaccinated SPF chickens demonstrated 62% and 96% protection against HHS, respectively (Pitcovski *et al.*, 2005; Shah MS., *et al.*, 2012). The 100k, penton, fiber-1, and subunit hexon vaccinations provide only moderate defense against HHS. More recent studies have demonstrated that SPF chicks immunized with the FAdV4 fiber-2 subunit vaccine had 90–100% protection against HHS. On the other hand, it was enhanced by raising the dosage of fiber and subunit antigen hexon (Chen *et al.*, 2018; Wang *et al.*, 2018). FAdV-(8b) serotypes causing IBH were found to be resistant to a recombinant FAdV fiber protein derived from a FAdV-8a strain. Homologous challenge dramatically lowered liver viral load in birds, but did not provide clinical protection against heterologous serotypes. Fiber immunization increased CD4 + T lymphocyte proliferation, moderated CD8 + T cell response, and prevented changes in systemic monocytes / macrophages and T cell sub-populations. This suggests that FAdV-E, a recombinant fiber, could be a vaccine candidate for controlling the harmful effects of homotypic infection (De Luca *et al.,;*2020). Recombinant viruses were created using specific genotype FAdV-4 strains that had the N-terminus of the fiber-2 gene deleted or fused to increase green fluorescent protein in order to reduce pathogenicity in SPF chickens. The main issue was that, compared to wild-type FAdV-4, in vitro recombinant virus titers were much lower (Xie *et al.*, 2021). A recombinant vaccination against FAdVs-IBH and -HHS, which were recently introduced in the poultry industry, has been developed through research. Shah *et al.* (2012) found that recombinant FAdV-4 structural proteins, fiber 2 or penton base, provide significant protection against HHS, whereas 100 K non-structural protein gives only 40% protection (Shah *et al.*, 2016).

2.9.4 Autogenous vaccines

To prevent and control disease in endemic areas, it is advised to deliver autogenous inactivated vaccinations based on the most common FAdV serotype isolates. Primary breeders with strict biosecurity standards employed autogenous inactivated vaccinations to minimize vertical transmission and transfer maternal immunity from parent flocks to progeny (Saifuddin *et al*.1990). In the USA, autogenous inactivated vaccinations are routinely utilized in main breeder flocks with strong biosecurity measures. Immunizing a grandparent stock at 10 and 17 weeks of age with an inactivated vaccine containing isolates of species FadV D and FadV E resulted in complete protection against IBH in breeders up to 50 weeks (Alvarado *et al.*, 2007). Prophylactic vaccination of broilers aged 10-15 days with a formalin-inactivated liver homogenate vaccine resulted in excellent field protection in Pakistan (Ahmad *et al.*, 2003). However, this successful autogenous vaccine has significant drawbacks, including batch-to-batch variation in viral antigen quantum, incomplete inactivation, and contamination by avian viruses, which could jeopardize biosecurity (Mughal *et al.*, 2011).

2.9.5 Role of maternal antibody on disease protection

IBH can be efficiently controlled by immunizing broiler breeder with a bivalent vaccination that combines live FAdV8a and FAdV11, according to a comparative research of unvaccinated and FAdV-vaccinated broiler breeders (Popowich *et al*., 2018).

CHAPTER-3

MATERIALS AND METHODS

3.1 Study design and ethical Approval

This cross-sectional study was conducted during 2020 to 2023. For this study breeder, broiler and commercial layer flocks were included. Three regions of Bangladesh were selected based on poultry population, density of commercial farms and disease prevalence. Mortality, morbidity and clinical findings suggested investigating the IBH disease and specific serotypes of virus. Liver & spleen of the dead birds were collected by postmortem examination. Few infected liver homogenates samples were inoculated into SPF embryonated chicken eggs for virus propagation. The suspected samples were preserved and processed for DNA extraction. Both real time PCR and conventional PCR were used for molecular characterization of causal agents. The PCR products were sent to sequence at National Institute of Biotechnology (NIB), Savar for detection of specific serotypes and Phylogenetic analysis. Blood serum samples were collected from live birds at beginning and after recovery of infection. For serological investigation of disease outbreak, Biochek indirect ELISA kits were used to detect the antibody titer level. However, before collecting blood, owners' verbal consents were obtained. The Institute of Research and Training (IRT) of Hajee Mohammad Danesh Science and Technology University (HSTU) granted ethical approval for this study under the number HSTU/IRT/ 2930 (1).

3.2 Study area

In Bangladesh, chickens are available over the entire nation. Huque and Khan (Huque *et al*, 2017) riven the country into three zones: A (105-1212 chickens per square kilometer), B (344-1108 chickens per square kilometer), and C (252-868 chickens per square kilometer) based on the density of chickens. This study includes Dinajpur Sadar upazila of Dinajpur district from zone A, Tetulia and Atwary upazilas of Panchagarh district from zone B and Gazipur Sadar upazila of Gazipur district from zone C (Figure 1).

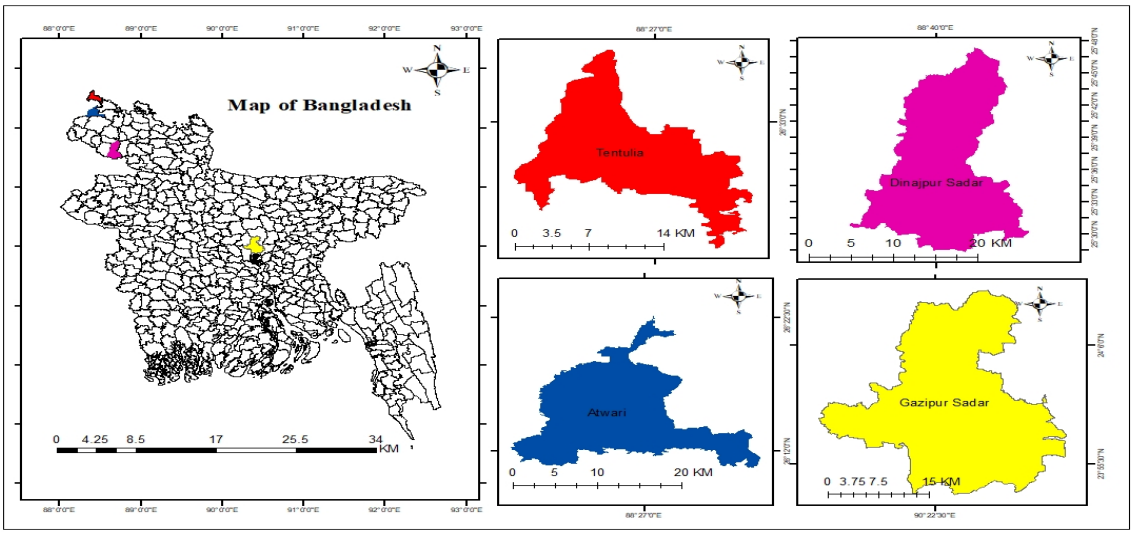


Figure 1: Location of the sampling Upazila of Bangladesh marked by different colors.

3.3 Objective -wise layout of this study. Objective 1

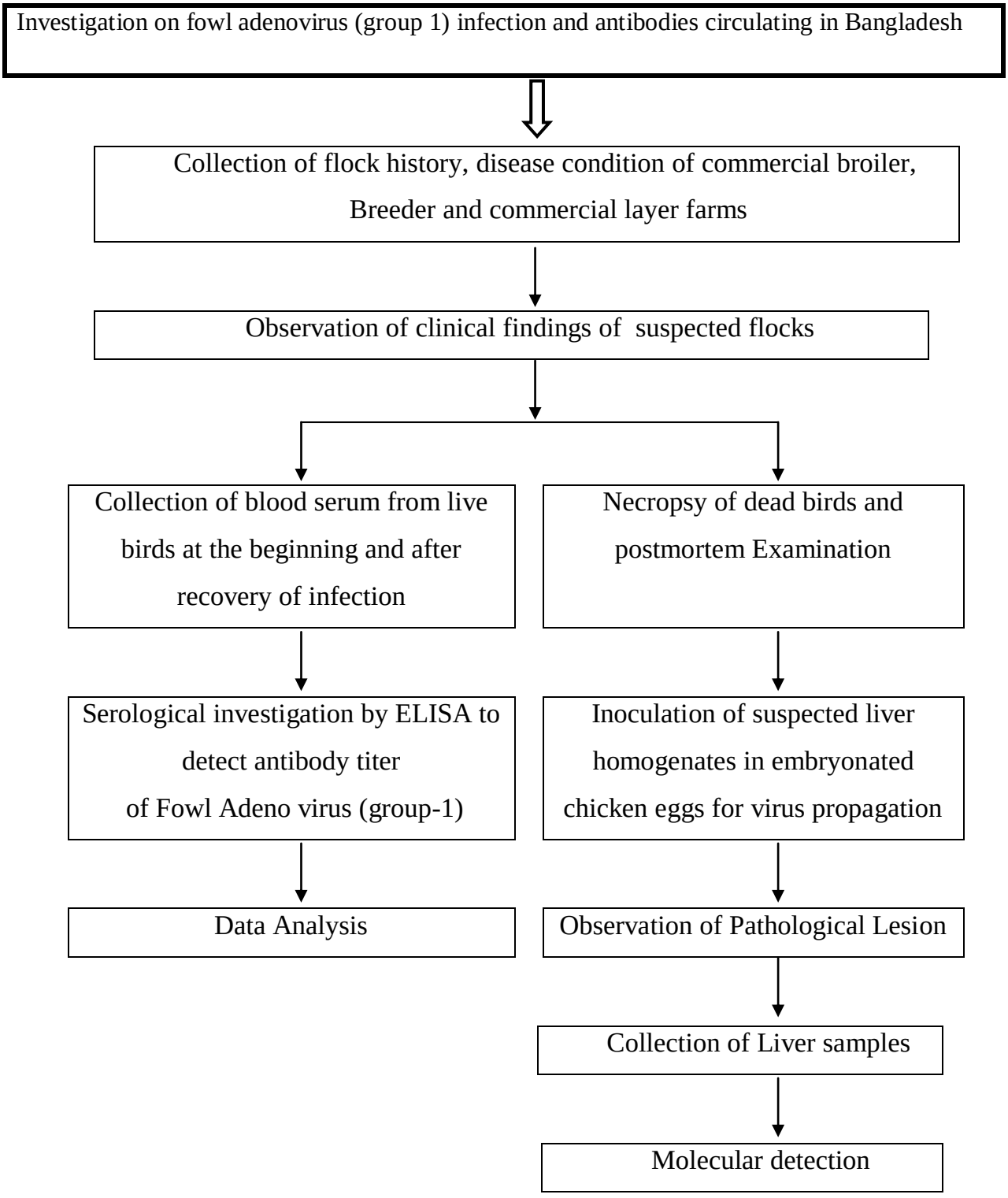


Figure 2: Flow diagram showing the schematic representation of investigation of Fowl Adenovirus

3.3.1 Flock History: A good history often provides clues that will help proper diagnosis. Information was taken on the type of bird, age, feed and water source and consumption rate, growth, morbidity and mortality, the description of the case, previous problems, vaccination program, and medicine being used.

3.3.2 Clinical findings: The general appearance of the birds was checked to determine which organ or system was involved in the disease. Increased sudden death, reluctant to move, depression and anorexia at early age suggested doing postmortem examination for diagnosis of Fadv related diseases (Cheema *et al.*, 1989).

3.3.3 Necropsy of Dead birds

3.3.3.1 ***Materials used for Necropsy***

Personal protective attire (Apron),

Disposable gloves

Knife

Tissue scissors

Forceps

Poultry shears

Plastic bags & sample cups

Culture swabs and transport tubes

Cotton swab

Plastic bags for disposal of carcass & contaminated supplies

Detergent or disinfectant solution

Bucket

Cooler box & ice packs

3.3.3.2 Necropsy procedure

During necropsy of dead birds both an external and internal examination of the carcass was done and a specific routine was followed to avoid missing of important lesions.

The postmortem examination was carried out of the birds that are representative of the problem in the suspected flocks.

The head, including the eyes, ears, nostrils, comb, wattles, mouth and beak were examined.

Across the upper beak and back into the sinuses was cut down; then through the mandible and down the esophagus into the crop.

The soft palate and larynx were examined and then the trachea was cut down and examined.

The abdomen and the vent were checked. The carcass was cut down between the abdomen and the legs and dislocated the hip joints. The skin was peeled off at the abdomen and breast. The breast was removed carefully by cutting through the abdominal muscles, ribs and coracoids. At this point, the thoracic and abdominal organs, paying particular attention to the air sacs, lungs, and liver were examined. Carefully the gizzard and intestines were raised and the abdominal air sacs, peritoneum, spleen were examined,

The circulatory and immune systems, the heart, pericardial sac, bursa, thymus, and lymphoid tissue of the thigh and intestine were examined.

The proventriculus was cut and removed the digestive tract and liver. The proventriculus, gizzard, and small and large intestines to the cloaca were opened and examined the lesions.

Then the kidneys and ureters were examined

Finally the skin, integument, muscles, bones and joints were examined.

3.4. Serological investigation

Anti-FAdV antibody was investigated by indirect ELISA (Philippe *et al.*, 2007).

3.4.1 Blood serum collection

3.4.1.1 Material used for blood serum sampling

Hand globes

Disposable syringes

Cotton & antiseptic solution

Eppen dorf tube (to store the collected serum)

Cooler box with ice packs (for transportation of samples to lab)

3.4.1.2 Blood serum sample collection procedure

3ml disposable syringe was used for blood collection.

Approximately 2.5 ml of blood was collected from wing vein for each serum sample.

The blood sample was not filled up to the top of the tube; space was left for serum separation.

Then the blood sample with in syringe was kept horizontally for 15-20 min.

The serum was separated by natural coagulation (2 hours at room temperature).

The clot was removed from sera and kept the serum samples at 4°C.

3.4.1.3 Description of blood serum samples

Besides dead birds, a total of 303 blood serum samples (156 samples at the beginning of suspected infection and 147 samples after recovery from

infection) were collected from suspected ten flocks for the anti-FAdV antibody analysis (Table-1) and stored at serology lab.

Table 1: Flock, status, age, number of serum samples and nature of test:

Flock type	Status	Age (days)	No. of sample	Test
Broiler (B1)	Beginning of exposure	9	14	ELISA
	After exposure	35	9	ELISA
Broiler (B2)	Beginning of exposure	7	14	ELISA
	After exposure	32	15	ELISA
Broiler (B3)	Beginning of exposure	11	14	ELISA
	After exposure	32	17	ELISA
Broiler breeder (BB1)	Beginning of exposure	14	18	ELISA
	After exposure	56	15	ELISA
Broiler breeder (BB2)	Beginning of exposure	9	18	ELISA
	After exposure	60	16	ELISA
Broiler breeder (BB3)	Beginning of exposure	8	18	ELISA
	After exposure	70	15	ELISA
Broiler breeder (BB4)	Beginning of exposure	14	15	ELISA
	After exposure	57	15	ELISA
Broiler breeder (BB5)	Beginning of exposure	16	15	ELISA
	After exposure	57	15	ELISA
Broiler breeder (BB6)	Beginning of exposure	16	15	ELISA
	After exposure	56	15	ELISA
Commercial layer (CL1)	Beginning of exposure	35	15	ELISA
	After exposure	70	15	ELISA

3.4.2 Serological test (ELISA)

3.4.2.1 Materials used at Serology laboratory

The laboratory was equipped with –

Freezer (for storage of blood serum)

Refrigerator

Glass Jar

Reagent reservoirs.

Micropipette (Single and multi channel)

ELISA Reader

Different sizes tip

Polystyrene micro titer plate.

Centrifuge machine

Lab stirrer.

Centrifuge machine

Air cooler

Measuring cylinder

Thermal Ice box

Autoclave devices.

Distillation plant

ELISA washer

Computer including Biochek ELISA software.

Procurement of reagents and accessories for research work was as follows: Biochek ELISA test kit, Hand gloves, antiseptic, syringes, test tubes, Different sizes tips, Eppendorf tube, tissue paper, cotton, distilled water.

3.4.2.2 Serology Laboratory preparation

The serology Laboratory was prepared before tests. At first, the personnel had worn clean apron, mask and hand gloves. The apparatus and lab accessories (which needed) were sterilized by steaming them to 121°C for 15 minutes under high pressure saturated steam autoclave machine. The micropipette, tips, micro titer plates were checked before used. The room temperature was 22°C. The serum sample, ELISA kits were kept at room temperature 1 hour before tested.

3.4.2.3 Used Antibody measurement kits

Serum samples were investigated for detection of antibody to FAdV by indirect ELISA using a commercial FAdV Group-1 antibody test kit (Biochek, Reeuwijk, Holland) following the manufacturer's instructions.

3.4.2.4 Description of the test

The FAdV ELISA kit quantified the quantity of FAdV antibodies present in chicken serum. Inactivated FAdV antigen was pre-coated on micro titer plates. Samples of diluted chicken serum were introduced to the micro titer wells containing any FAdV antibodies that may bind and create an antigen-antibody complex. Following that, additional serum proteins and non-specific antibodies were removed by washing procedure.

The wells were then filled with anti-chicken IgG that had been labeled with the enzyme alkaline phosphatase. This antibody binds to any chicken anti-FAdV antibodies that were initially attached to the antigen. Substrate in the form of pNPP chromogen was added after a second wash to eliminate the unreacted conjugate. If anti-FAdV antibody was present, a yellow color formed, and the amount of anti-FAdV directly correlated with the color's intensity.

3.4.2.5 Reagents of Biochek FAdV ELISA Kit

FAdV antigen Coated plate: Inactivated viral antigen on micro titer plates.
Conjugate reagent: Anti-Chicken Ig, Alkaline Phosphatase in Tris buffer with protein stabilizers, inert red dye and sodium azide preservative. (0.1% w/v).
Substrate tablets: PNPP (p-Nitrophenyl Phosphate) tablets to dissolve with substrate buffer
Substrate buffer reagent: Diethanolamine buffer with enzyme co-factors.
Stop solution: Sodium hydroxide in Diethanolamine buffer.
Sample diluents reagent: Phosphate buffer with protein stabilizers and sodium azide preservatives (0.1% w/v).
Wash buffer: Powdered phosphate buffered saline with Tween.
Negative control: Specific Pathogen free serum in Phosphate buffer with protein stabilizers and sodium azide preservative (0.1% w/v).
Positive control: Antibodies specific to FAdV in phosphate buffer with protein stabilizers and sodium azide preservatives (0.1% w/v).

3.4.2.6 Materials and Equipment required for FAdV ELISA at Serology Lab.

Micro Pipettes (single and multi channel).
Disposable tips.
Micro titer plate for sample dilution.
Distilled water.
ELISA plate reader with 405 nm filter.
Micro titer plate washer.
Reagent reservoir.
Tips holder.
Absorbent paper/towel.

3.4.2.7 Reagent preparation

Substrate reagent: Two tablets were added to 11 milliliters of substrate buffer and mixed until completely dissolved to create the substrate reagent. The produced reagent should be created the day it is needed, although if stored in the dark at +4°C, it will remain stable for a week.

Wash buffer: The contents of one wash buffer sachet were added to one liter of distilled water and mixed until completely dissolved.

The remaining kit components were all ready to use; however, let them an hour to reach room temperature (22 °C).

3.4.2.8 ELISA Test procedure

A polystyrene micro titer plate was used for the dilution plate, and each well held 5 µl of serum sample.

In each well of the dilution plate, 245 µL of Biochek Green sample diluents was introduced. As a result, the serum was diluted 1:50 with the diluents in the dilution plate.

After taking the FAdV antigen-coated plate out of the sealed bag, the samples' locations were noted on the template.

Each well on a Biochek test plate received 50 µL of sample diluents (Green) (excluding well of neg. and pos.).

The solution was drawn into the pipette and then released back into the well to mix the 1:50 serum dilution with the pipette. The steps were repeated four times.

Subsequently 50 ul of the 1:50 diluted serum from the dilution plate was added to each corresponding well of the Biochek test kit plate. As a result, the Biochek test plate had a final 100 µL / well of 1:100 serum dilutions.

Wells A1 and B1 were filled with 100 µL of Negative Control.

Wells C1 and D1 were filled with 100 µL of positive control.

After placing a lid on the plate, it was incubated for half an hour at room temperature (22 °C).

The contents of each well were aspirated by 350 µL of wash buffer for four times. The plate was inverted and taped firmly on absorbent paper.

100 µL of conjugate reagent was added into the appropriate wells. The plate was covered with lid and incubated at room temperature (22-27°C) for 30 min.

The contents of wells was aspirated and washed 4 times with wash buffer (350 µL per well). The plate was inverted and taped firmly on absorbent paper.

The relevant wells were filled with 100 µL of the substrate reagent. The plate was incubated for fifteen minutes at room temperature (22–27°C) with a lid on.

The reaction was stopped by adding 100 µL of stop solution into the relevant wells.

Microtiter plate reader was blanked on air and the absorbance of samples and controls were recorded by reading at 405 nm.

3.4.2.9 Interval of Titer Measurement

Antibody titer of FadV of each flock was measured by indirect ELISA at beginning of disease and after recovery of infection and recorded accordingly.

3.4.2.10 Calculation of Results

The FadV positive control was carefully calibrated to accurately reflect the considerable level of FadV antibody detected in chicken serum. The positive control then be used to calculate the relative number of antibodies in the serum samples. The S/P ratio, or sample to positive ratio, is used to express this relationship.

The difference between the mean absorbance of the negative control and the positive control should be more than 0.15 and the mean absorbance of the negative control should be less than 0.3 for the test result to be valid.

3.4.2.11 Interpretation of results

Mean of negative control OD: (Well A1 +Well B1)/2

Mean of Positive control OD: (Well C1 +well D)1/2

Calculation of S/P ratio:

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

Calculation of Antibody titer:

The following equation relates the S/P of a sample at a 1:100 dilution to an end point titer.

$$\log_{10}\text{Titer} = 1.1 * \log_{10}\left(\frac{S}{P}\right) + 3.361$$

Samples with an S/P ratio of 0.5 or greater was considered as positive for anti-FAdV antibody. In other words samples having titer 1071 or greater is considered as positive.

3.4.2.12 Software program developed by Biochek

Biochek ELISA software version Biochek 2020.0.0.1 were used. This software contained data (positive control, negative control, s/p ratio and OD value) which were adjusted in such a way that when OD value of the samples that were obtained from ELISA reader were fed, the antibody titer of the samples obtained automatically. To get the antibody titer of all 92 samples in the particular plate, positive control and negative control would be kept fixed in the program only for that particular plate.

3.4.3 Statistical analysis of data: The Statistical Package for Social Science (SPSS) version 20 was used to analyze the collected data. The descriptive results were presented as mean and standard error. Unpaired T-test was used for two variables (BE: Beginning of Exposure and AE: After Exposure). The differences were considered statistically significant at p values < 0.01 or < 0.05.

3.5 Isolation of FAdV in Embryonated chicken eggs

3.5.1 Material used

SPF embryonated chicken eggs, Sterilized syringe, Phosphate buffer saline, penicillin, streptomycin, filter (0.22 μm), and incubator.

3.5.2 Procedure

Tissue samples, consisting of liver were homogenized in phosphate-buffered saline (pH 7.2) with 1:10 w/v ratio. The homogenates were kept at -80^{o}C for 2 hours and thawed at room temperature for 30 min. Freezing and thawing cycle was repeated for three times and finally centrifuged at 8000×g for 3 min. Supernatant was collected and filtered.

The method used to propagate the virus involved inoculating by chorioallontoic route (Winterfield *et al.,* 1973) of three 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs (one egg for each sample from a particular suspected flock B1, BB2 and BB3) with 0.2 ml of filtered suspension (20% W/V) of pooled liver tissue homogenate samples. Also 200 μg/ml of penicillin and 100 μg/ml of streptomycin were added in the inoculums. The inoculated SPF embryonated chicken eggs were incubated at 37°C and monitored for 7 days. One embryonated SPF chicken egg (aged 10 days), was used as the negative embryo control. It was inoculated with 0.2 ml of sterile phosphate-buffered saline. Mortalities in embryos that happened before 48 hours were considered non-specific causes and were eliminated by candling. Every embryo that died after 48 hours of inoculation were harvested and necropsied. Liver samples were examined for pathological lesions and collected for molecular detection.

3.6 Objective -wise layout of this study. Objective 2

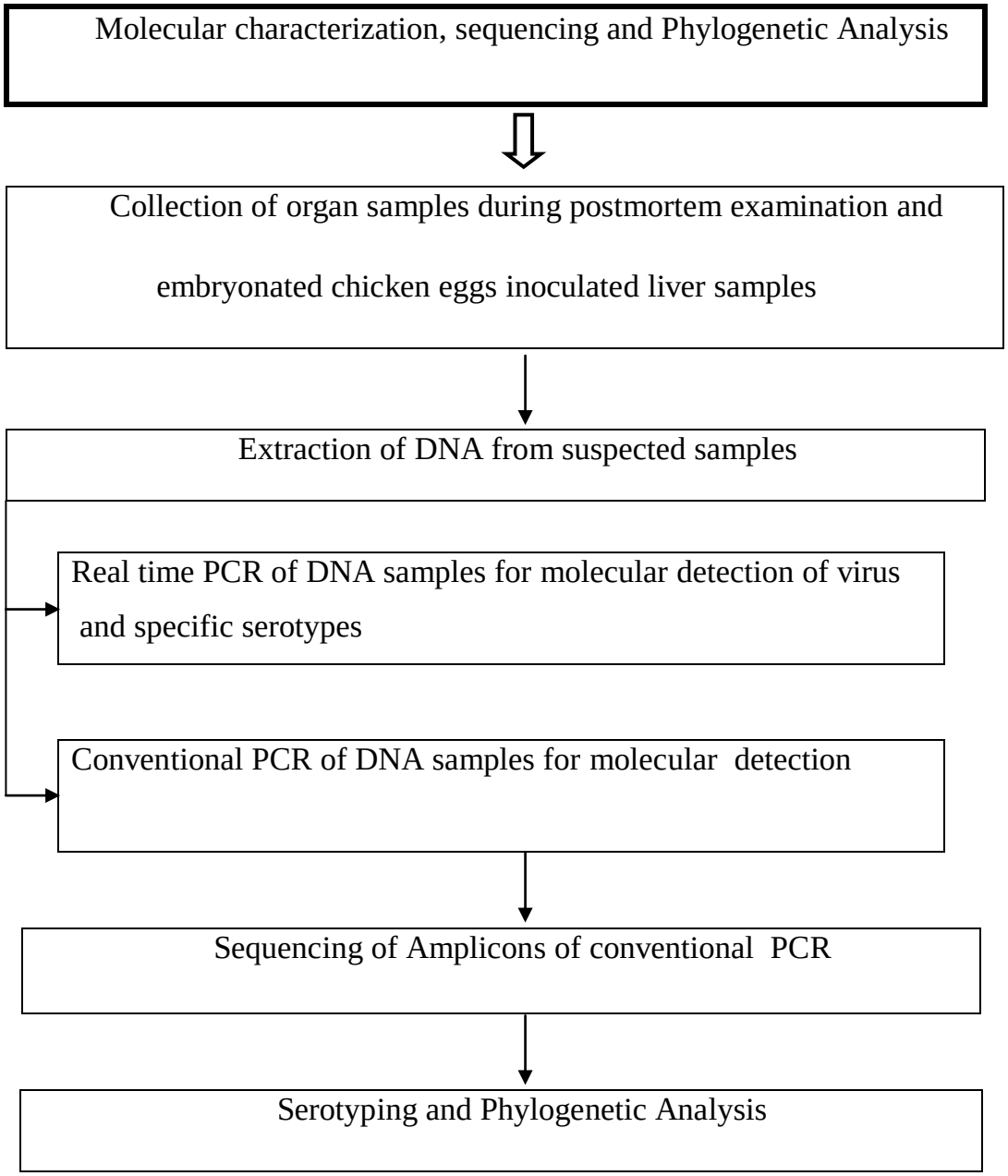


Figure 3: Flow diagram showing the schematic representation of molecular detection, sequencing, serotyping and Phylogenetic analysis.

3.6.1 Materials used at Molecular lab

The Molecular laboratory was equipped with –

Freezer -20 ^{o}C and -80 ^{o}C (Storage of organ and DNA samples)

Refrigerator (for molecular test reagents eg. Master-mix)

Minispin (For proper mixing of prepared samples)

Micropipette (Single and multi channel for sample and reagents)

High speed centrifuge capable of centrifuging>10,000×g

DNase, RNase free Dual filter Tips

Eppen dorf tube

Water bath

PCR cabinet

Vortex (for mixing sample and reagents properly)

Pipette Stand

Air cooler

Measuring cylinder

PCR Tube Rack

Autoclave devices.

Distillation plant

Electronic balance

Biosafety Cabinet

Gel electrophoresis machine

Gel documentation system with computer

Thermo cycler (For conventional PCR and for realtime PCR)

3.6.1.1 Consumable Materials & reagents used at molecular lab

Sterile, Dnase free 1.5-ml microcentifuge tubes for elution

Microtips (2.5 µl, 10 µl, 100 µl and 1000 µl)

Lysates (samples)

Distilled water

Hand globes, mask and tissue paper

96-100% ethanol

Reagents (rtPCR kits, Primers, Probe, Mastermix)

3.6.2 Collection of organ samples for molecular test

A total of ten flocks of five different farms located in geographically distant three locations viz. Gazipur Sadar Upazila, Dinajpur Sadar upazila, Tetulia and Atwar upazilas of Panchagarh district, were included in this study. The flocks include were one commercial layer flock (5000 birds) from Dinajpur district, four broiler breeder flocks (10,000 birds/flock) from Panchagarh district and three commercial broiler flocks (2000 birds/flock) and two broiler breeder flocks (10,000 birds/flock) from Gazipur district (Table 2 and Figure 1). From each flock five samples were collected. Each sample composed of liver (~2gm) and whole spleen of a suspected dead chicken. So, from ten flocks a total 50 samples were obtained for virological investigation (Table 2). The liver samples of inoculated embyo also were collected. The collected organ samples from broiler, broiler breeder and commercial layer of study area preserved in the Molecular Laboratory at -80^{o}C.

Table 2: Flock type, size, location, age, and organs of collected sample:

Flock Type	Flock size	Location	Age (days)	Organs
Broiler (B1)	2,000	Gazipur Sadar	9	liver and spleen
Broiler (B2)	2,000	Gazipur Sadar	7	liver and spleen
Broiler (B3)	2,000	Gazipur Sadar	11	liver and spleen
Broiler breeder (BB1)	10,000	Gazipur Sadar	14	liver and spleen
Broiler breeder (BB2)	10,000	Gazipur Sadar	9	liver and spleen
Broiler breeder (BB3)	10,000	Atwary, Panchagarh	8	liver and spleen
Broiler breeder (BB4)	10,000	Atwary, Panchagarh	14	liver and spleen
Broiler breeder (BB5)	10,000	Tetulia, Panchagarh	16	liver and spleen
Broiler breeder (BB6)	10,000	Tetulia, Panchagarh	15	liver and spleen
Commercial layer (CL1)	5,000	Dinajpur Sadar	35	liver and spleen

***3.6.3 DNA (deoxyribonucleic acid) extraction*:** Tissue samples, consisting of liver and spleen, were homogenized in phosphate-buffered saline (pH 7.2) with 1:10 w/v ratio. The homogenates were kept at -80^{o}C for 2 hours and thawed at room temperature for 30 min. Freezing and thawing cycle was repeated for three times and finally centrifuged at 8000×g for 3 min. Supernatant was collected and used for DNA extraction. PureLink genomic DNA Mini Kit (Invitrogen, USA) was used to extract DNA as per manufacturer's instruction. The purity of the extracted DNA was checked by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and preserved at -20^{o}C until further analysis.

3.6.3.1 Materials supplied with DNA extraction kits

PureLink^{R} Genomic Wash Buffers 1 and 2, PureLink^{R} Genomic Digestion PureLink^{R} Genomic Lysis/Binding buffer, Proteinase K, RNase A

PureLink^{R} Genomic Elution Buffer

PureLink^{R} Spin Columns in collection Tubes

PureLink^{R} collection Tubes.

3.6.3.2 DNA Extraction procedure

Water bath was fixed at 55ºC.

The homogenates of suspected organ sample was centrifuged by high speed centrifuge machine at 8000×g for 1 min.

After centrifugation the cell pellet was resuspended in 180 µL Digestion Buffer.

20 µL Proteinase K was added to lysis the cells and mixed by vortexing.

The tube was incubated at 55ºC with occasional vortexing until lysis is complete (approximately 45 min)

20 µL RNase A was added to the lysate (sample), mixed by vortexing and incubated at room temperature for 2 min.

200 µL Lysis/Binding buffer was added and mixed well by vortexing to obtain homogenous solution.

200 µL 100% ethanol was added to the lysate and mixed well by vortexing for 5 sec to obtain homogenous solution.

The sample (around 640 µL) prepared with Lysis/Binding Buffer was taken in spin column and centrifuged at 10000×g for 1 min at room temperature.

The collection tube was discarded and placed the spin column into a new collection tube.

500 µL Wash Buffer 1 prepared with ethanol was added to the column and centrifuge at 10000×g for 1 min at room temperature. The collection tube was discarded and placed the spin column into a new collection tube.

500 µL Wash Buffer 2 prepared with ethanol was added to the column and centrifuged at maximum 12000×g for 3 min at room temperature.

The collection tube was discarded and placed the spin column in a sterile 1.5ml micro centrifuge tube.

100 µL Elution Buffer was added to the column and incubated at room temperature for 1 min.

The column was centrifuged at maximum 12000×g for 1 min at room temperature.

The column was discarded and the micro centrifuge tube contained purified genomic DNA.

3.6.4 Detection of FAdVs by molecular test

3.6.4.1 Real-time PCR

Materials and procedure

Samples were principally tested by realtime PCR (rt-PCR). Real time PCR was done for detection and serotyping of FAdV isolates. In case of rt-PCR, Taqman chemistry based FAdV Pockit kit (GeneReach Biotechnology Corp, Taiwan) was used for detection of all Species (FAdV-A,B,C,D,E) & 12 serotypes as a whole, and serotype FAdV-4 and serotype FAdV-8b by serotype specific kits. This PCR was carried out on a 7500 FAST Real-Time PCR System (Applied Biosystems) with ABI 7500 software (Version 2.3) besides PCRmax Eco 48 realtime PCR machine. The reaction was set in 25 µL scale and consisted of

2μL template DNA and 23μL of premix reagents. The thermal profile consisted of the 40 cycles of at 95°C for 3min, at 93°C for 15 sec and at 60°C for 1min. However, samples were also tested by conventional PCR for serotyping (5, 11) and further Phylogenetic analysis.

3.6.4.2 Conventional PCR

3.6.4.2.1 Primer used

In case of conventional PCR, fragment of loop 1 (L1) region of the *hexon* gene, amplicon size 590 bp of FADV was amplified by using the primers-

F:5′ ATGGGAGCSACCTAYTTCGACAT-3′ and

R: 5′-AAATTGTCCCKRAANCCGATGTA-3′ (Raue *et al*., 2005).

3.6.4.2.2 Composition of conventional PCR reaction

The PCR reaction mixture was in a volume of 25 μL containing 12.5 μL of master mix (Thermo Scientific, Waltham, MA, USA), 2 μL (10 μM) of each forward and reverse primer, 6.5 μL of nuclease free water and 2 μL of template DNA. Negative control (all reagents except test sample) was run along with test sample and total volume was adjusted with nuclease free water. Amplification was carried out in a 2720 thermal cycler (Applied Biosystems, UK).

3.6.4.2.3 Thermal profile of conventional PCR

The thermal profile was at 95°C for 10 min for initial denaturation followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 30 sec, and extension at 72°C for 45 sec, and a final extension at 72°C for 5 min.

3.6.4.2.4 Agarose Gel Electrophoresis procedure

Amplicons of conventional PCR were examined in an ultrapure 1.5% agarose gel (Invitrogen) with SYBR Safe DNA gel nucleic acid stain (Thermo Fisher Scientific) using 1X TAE electrophoresis running buffer. The gel casting tray

was put together using a gel comb with the right amount and size of teeth. A 1.5% agarose solution was made by melting it in a microwave oven with TAE (1X) buffer. Then poured into the casting tray and let to set on the bench, Sybr-safe DNA gel dye (Invitrogen, USA) was added along with melted agarose. A thermoscientific DNA loading dye was added with each PCR product samples.

The gel tray with the firm gel inside was moved to the electrophoresis tank with enough TAE (1X) buffer in it to cover the gel by 1 mm. The comb was carefully taken out. 5 µL of every PCR product samples were added to the corresponding gel well. One well contained 5 µl of a 100 bp size ready-load DNA marker (thermoscientific, Lithuania). The power supply and the electrophoresis were linked to the leads of the electrophoresis equipment at a voltage of 100 volts. The power supply was turned off after the DNA had sufficiently moved, as determined by the loading buffer's bromophenol blue migration. Then, the gel was put on the UV trans-illuminator (UVITEK, USA) in the image documentation system's dark chamber. After turning on the system's UV light, the picture was focused, captured, and saved to a USB flash drive.

3.7 Sequencing

Materials and methods

Sequencing was done with Applied Biosystems 3130 Genetic Analyzer using 20 µL reaction volume containing approximately 10 ng purified PCR product as template, 5X ready reaction premix 4.0 µL, Big Dye terminator buffer 2.0 µL, primer 0.32 µL and ultrapure water to make 20 µL. The primers used for amplification was also used for sequencing. The sequence was read from both directions. The electropheregram analysis and multiple sequence alignment (MSA) were done using MEGA-11 software (Tamura *et al.*, 2021).

***3.8 GenBank accession number*:** Nucleotide sequences generated in this study were submitted to GenBank and obtained accession number (Table 3)

Table 3: Sample ID & accession number of the sequences

Name of Sample ID	Accession number
Fadv/ Gazipur/ Bangladesh/G 01/2021	OP454906
Fadv/ Panchagarh/ Bangladesh/T 02/2021	OP454907
Fadv/ Panchagarh/ Bangladesh/A 03/2022	OP454908
Fadv/ Gazipur/ Bangladesh/G 09/2021	OP502745
Fadv/ Gazipur/ Bangladesh/08714/2022	OP004931
Fadv/ Gazipur/ Bangladesh/G 06/2021	OP502746

3.9 Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method and Tamura-Nei model (Tamura *et al.*,, 1993). Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*, 2021).

Plate 1: Depression, ruffled feath, reluctant to move of 1.3wks broiler breeder

Plate 2: Materials of postmortem examination and sample collection

Plate 3: Cool box for transportation of samples

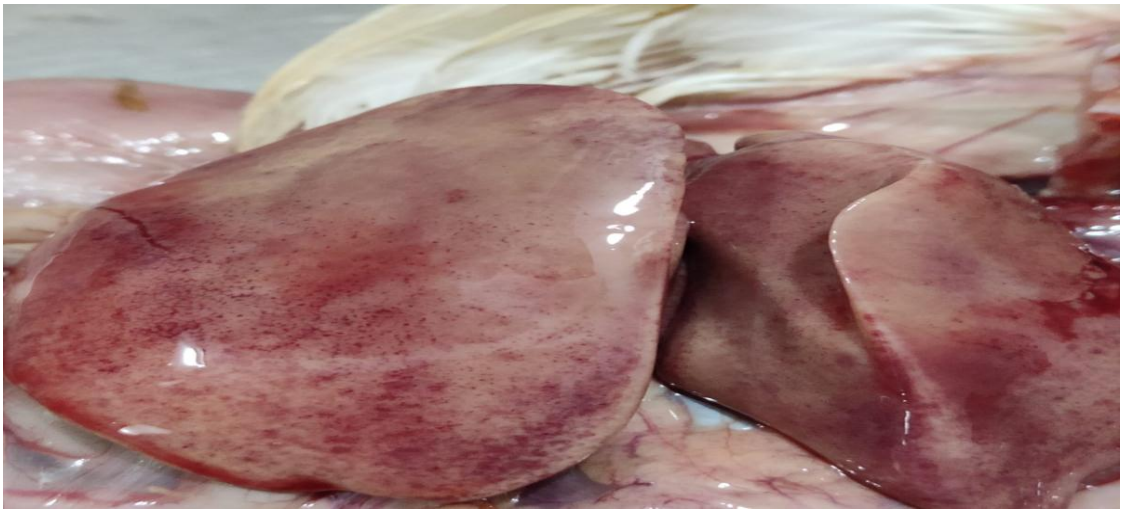


Plate 4: Hemorrhagic, pale and enlarged liver of 7 days old broiler

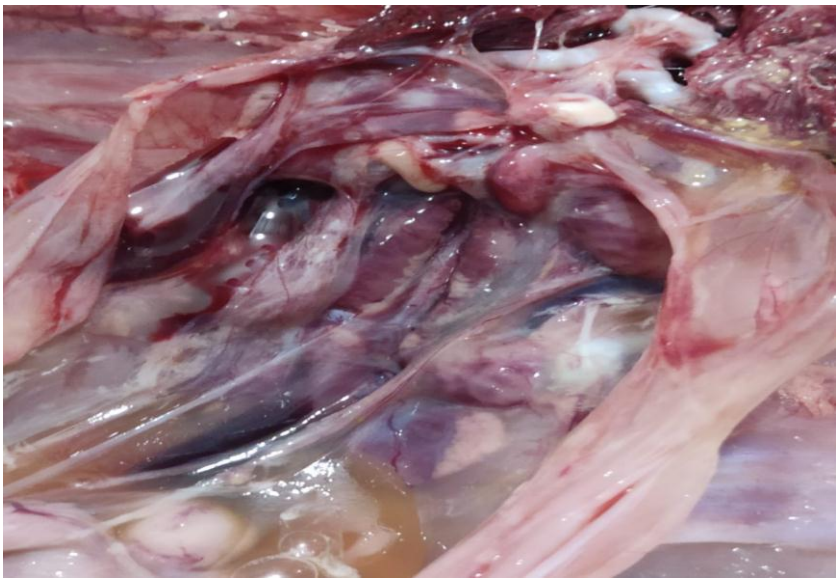


Plate 5: Swollen and hemorrhagic kidney of 10 days old broiler breeder

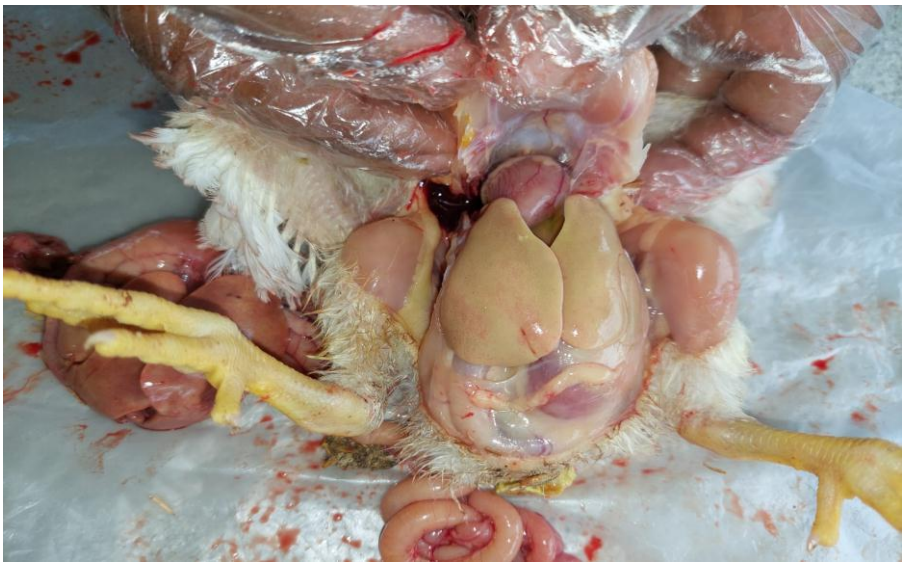


Plate 6: Tan colored liver of 7 days old broiler

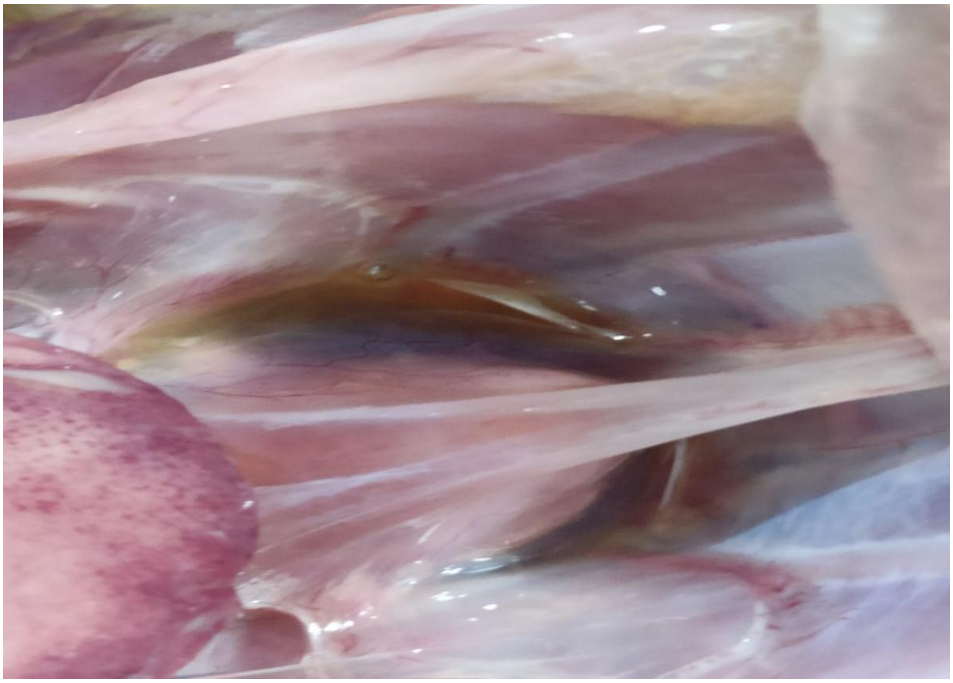


Plate 7: Hydropericardium of 12 days old broiler

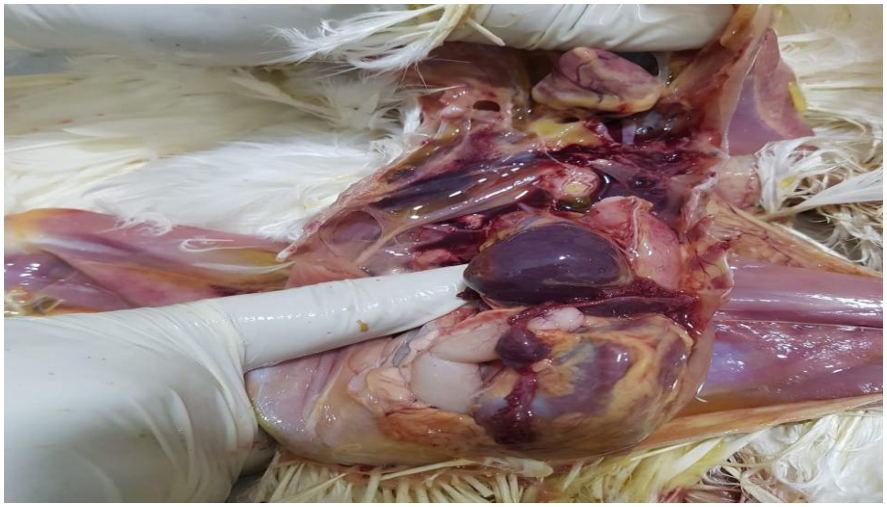


Plate 8: Swollen and congested spleen of 15 days old broiler breeder

Plate 9: Homogenates liver samples for ECEs inoculation

Plate 10: Embryonated egg inoculation of infected organ homogenates

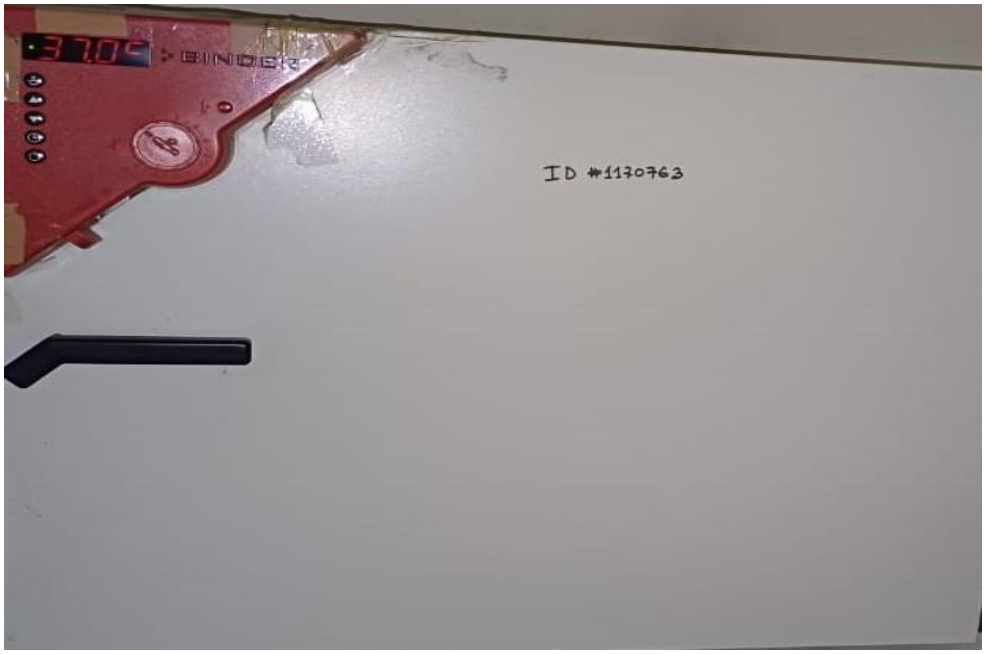


Plate 11: Incubator (temperature 37^{o}C)

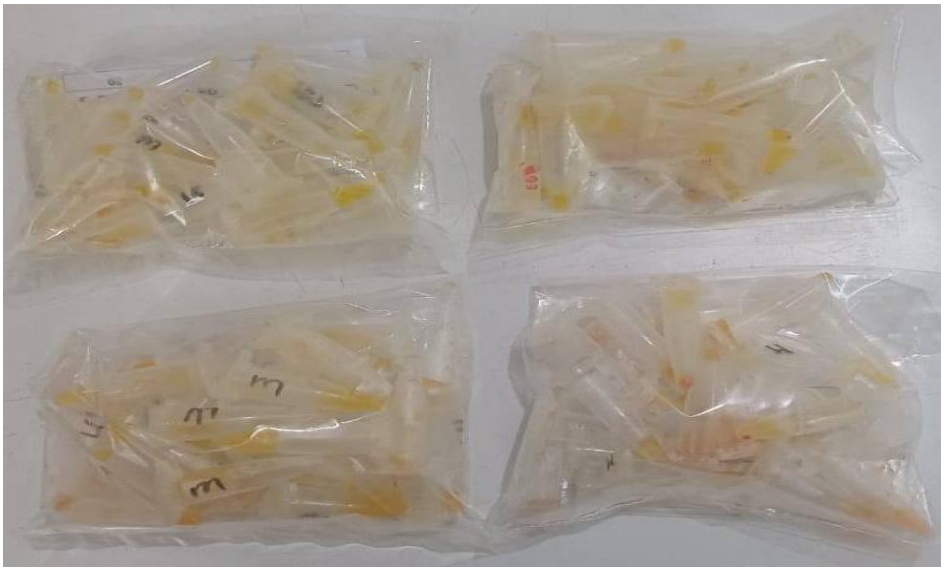


Plate 12: Blood serum samples

Plate 13: Multi and Uni channel pipette, tips for ELISA

Plate 14: Fadv ELISA plate & reagents

Plate 15: Diluted samples & control on antigen coated plate

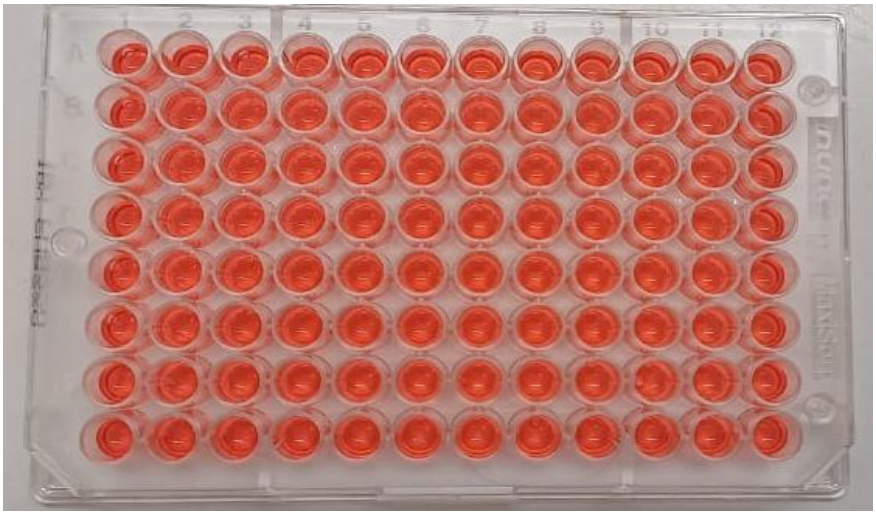


Plate 16: Conjugate on antigen coated plate

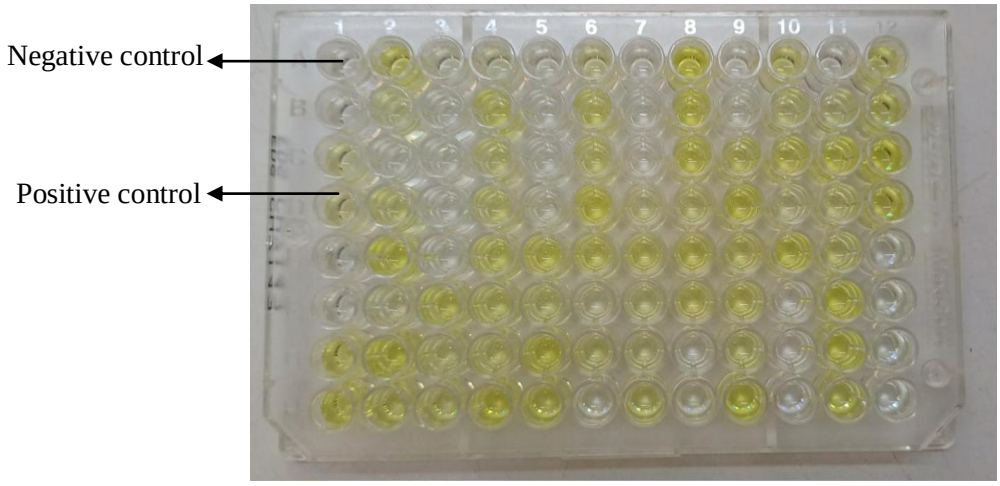


Plate 17: Substrate on antigen coated plates

Plate 18: Taking reading by ELISA reader

Plate 19: Pooled organ samples of liver and spleen for DNA extraction

Plate 20: Putting sample at high speed centrifuge machine to homogenize

Plate 21: Incubation of homogenates at 55°C

Plate 22: DNA extraction kits

Plate 23: Adding Digestion buffer with sample pellet

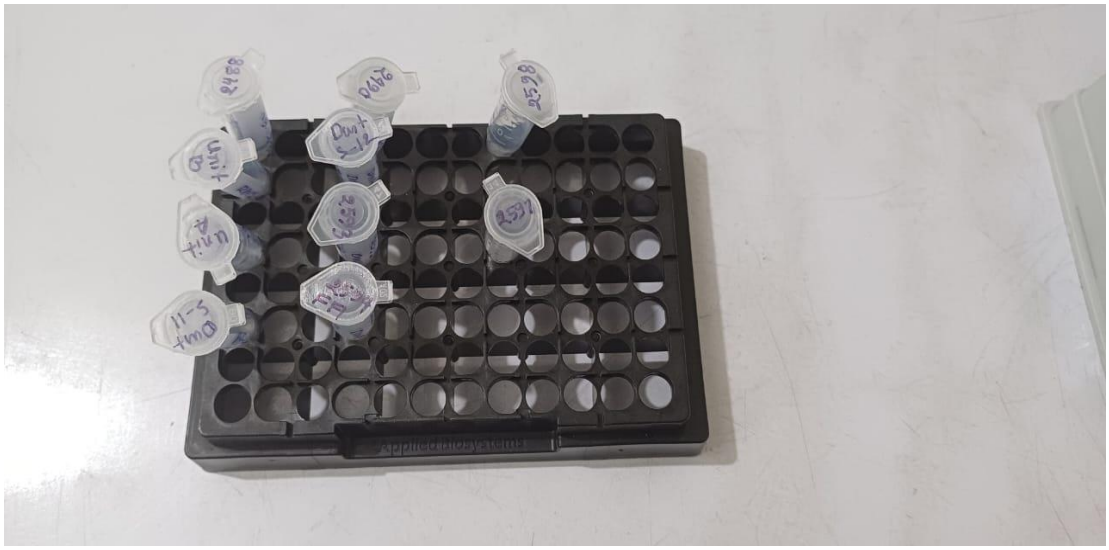


Plate 24: Extracted DNA samples

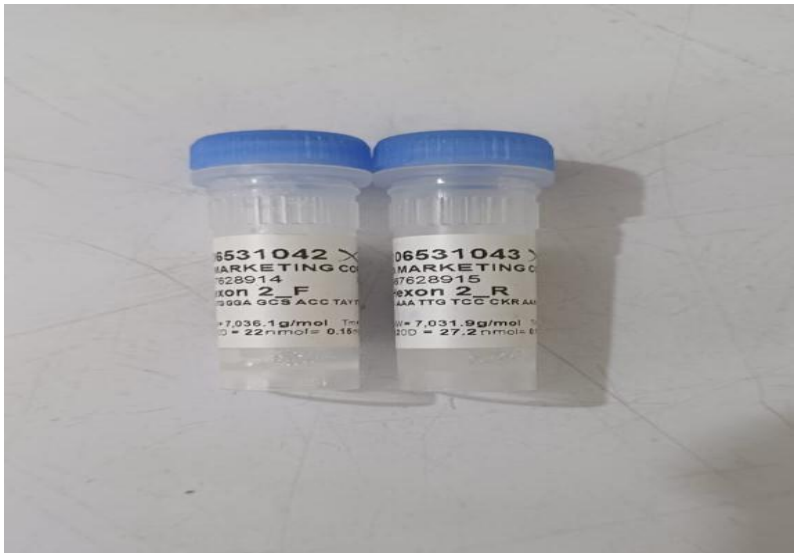


Plate 25: Forward & Reverse primers

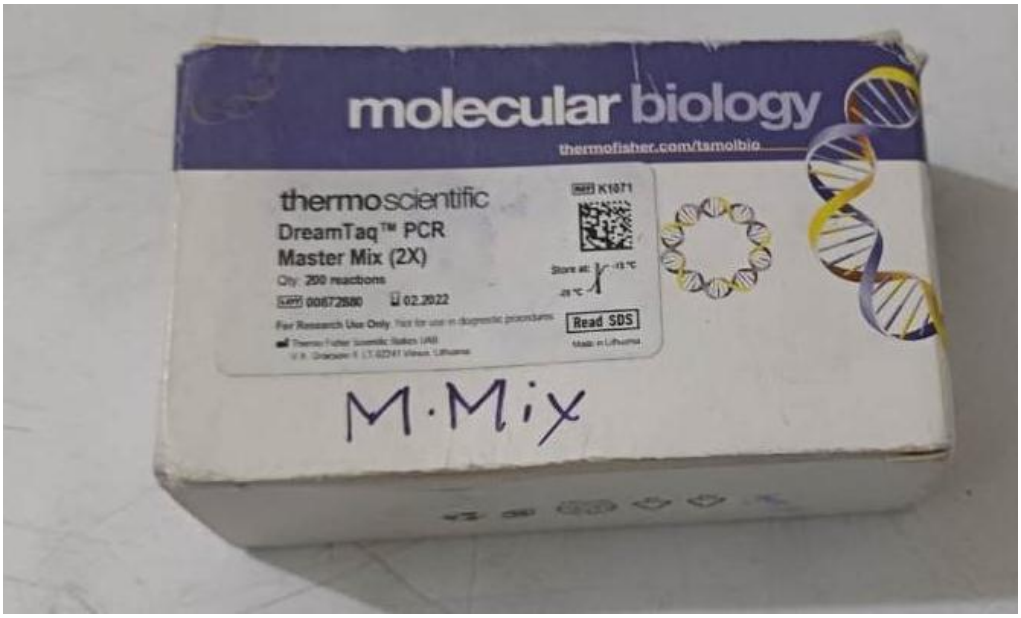


Plate 26: Master-mix for PCR

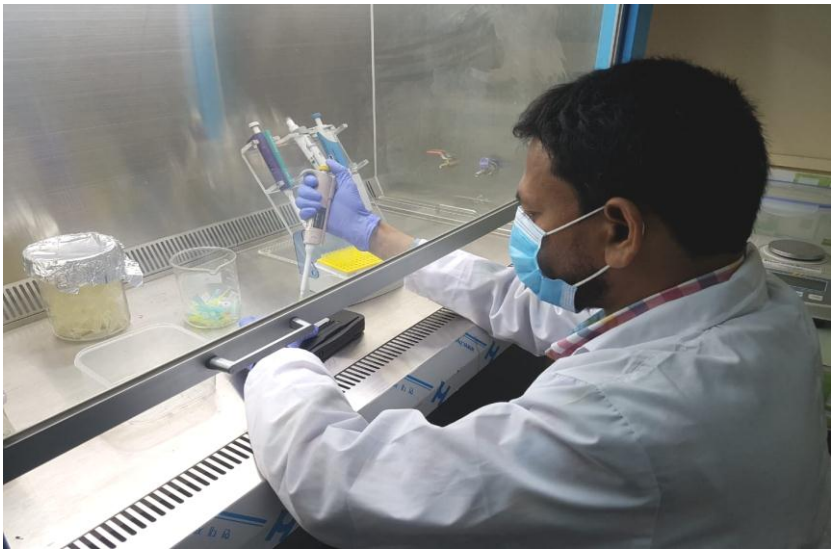


Plate 27: Sample preparation for Real-time PCR using Biosafty cabinet

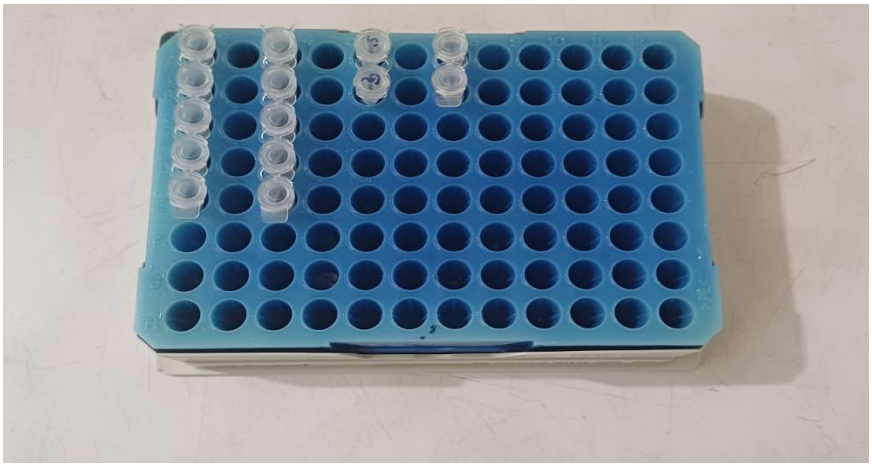


Plate 28: Ready PCR samples with reagents for amplification

Plate 29: Amplification, in PCRmax Eco 48 realtime PCR machine

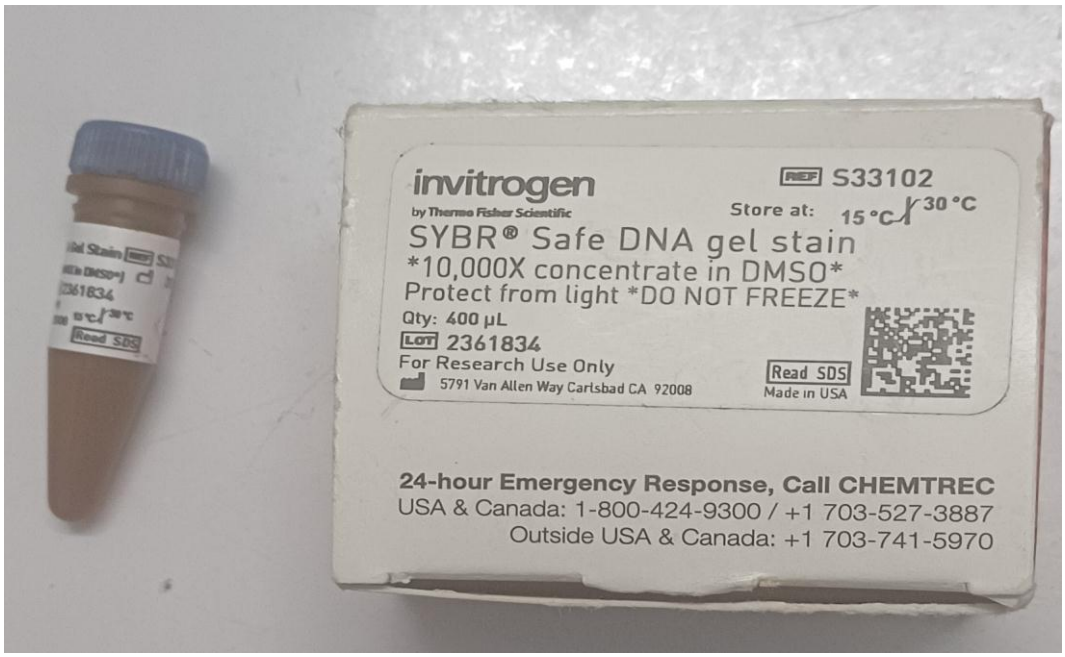


Plate 30: Sybr safe DNA gel Stain.

Plate 31: UltraPure™ Agarose

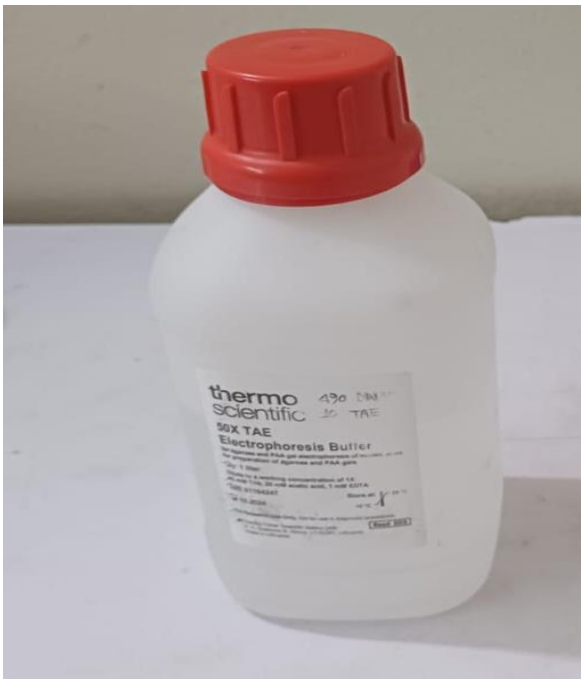


Plate 32: 50X TAE Electrophoresis buffer

Plate 33: GeneRular 100 bp plus DNA Ladder & SYBR Safe DNA gel nucleic acid stain (TermoFisher Scientifc)

Plate 34: Conventional PCR model ABIS 2720

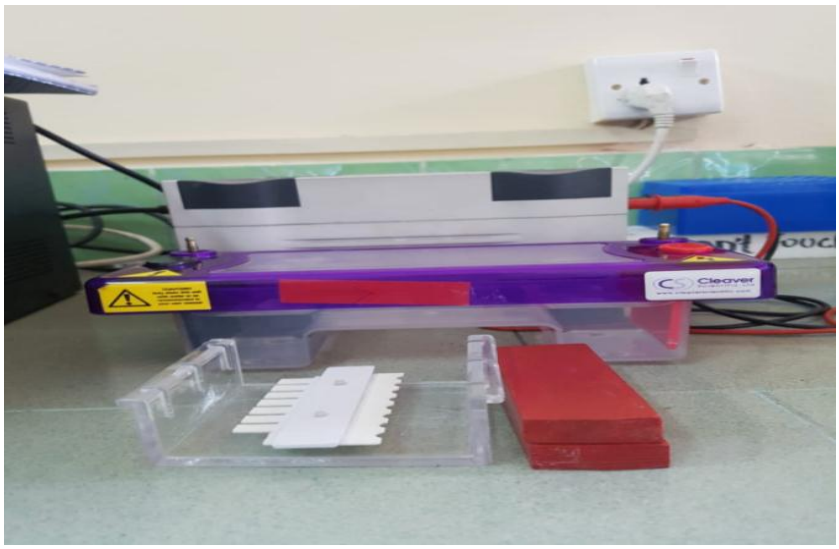


Plate 35: Electrophoresis power supply

Plate 36: Gel documentation

CHAPTER-4

RESULTS

4.1 Clinical findings: Ten flocks of broilers (*n=3*), broiler breeders (*n=6*) and commercial layer (*n=1*) from five farms of three geographically distant locations, Gazipur, Dinajpur and Panchagarh districts of Bangladesh were investigated. Veterinary practitioners and farmers reported that they observed drowsiness, huddling with ruffled feathers, inappetence (plate 1), stooping, mucoid droppings, high mortality in their farms and taken veterinary care with history of no response to treatment, even with different antibiotics. Broiler flocks B1, B2 and B3 were derived from one farm and broiler breeder flocks BB1 and BB2 were derived from another farm in the Gazipur district. On the other hand broiler breeder flocks BB3, BB4 and BB5, BB6 were originated from the Atwary and the Tetulia upazila, respectively in the Panchagarh district. Distances among the multiple flocks of each of the farms were within 50-80 feet. Only single commercial layer flock (CL01) derived from Dinajpur sadar upazila. Clinical signs of anorexia, lethargy, reluctant to move, drowsiness, reduced body weight gain, lack of uniformity, respiratory distress, in chicks of up to five weeks of age were also noticed during farm visit. Among the studied flocks (*n=10)* morbidity was found 10%-65% whereas mortality varies from 5% to 30%. However, morbidity and mortality in FAdV positive flocks were within 23%-65% and 12%-30%, respectively. Higher morbidity (65%) and mortality (30%) was found in a broiler breeder flock of Tetulia upazila, Panchagarh and serotype 11 was detected from that flock. Flocks affected with serotype 8b had 23%-45% morbidity and 15%-25% mortality among the flocks. Lowest morbidity (10%) and mortality (5%) was found in commercial layer chicken flock of Dinajpur Sadar. However, FAdV was not detected from this flock. Comparatively, higher morbidity (35%-65%) and mortality (12%-30%) was found in broiler breeder flocks than broiler flocks where morbidity 25%-30% and mortality was 15%-20%, respectively. The mortality reduced gradually 2-3 weeks later.

4.2. Postmortem lesion: Hemorrhages at skeletal muscle, an enlarged pale, friable liver with a yellow to tan color that was mottled with a localized soft area, and even petechial and ecchymotic hemorrhages beneath the capsule and parenchyma were discovered during the postmortem examination. Accumulations of fluid in pericardial sac were also found in one case. Swollen and hemorrhagic kidneys were frequent, the enlarged congested spleen and pancreas were also noticed (plate 4-8).

4.3 Anti-FAdV antibody prevalence: Anti-FAdV antibody was investigated by ELISA (Calnek *et al.,* 1982) in serums collected at the beginning of suspected infection (*n=156*) and after recover from infection (*n=147*) and results are presented in Table 4. Irrespective of sampling time, the anti-FAdV antibody was found in 9 flocks at the beginning of infection and after recovery of infection. Samples originated from commercial layer flock of Dinajpur Sadar upazila was found negative for anti-FAdV antibody (Fig 26, 27). At beginning of infection only 0-40% samples were found seropositive and their titer was very low (399±81-885±172).On the other hand after recovery of infection 53.33-100% samples were found seropositive and their titer was found very high and ranges from 1246±172-9699±832 (Table 4 and Fig.8-25, 28). The proportional seroprevalence at sample level was higher (89.12%; 131/147) in case of samples obtained after recovery from suspected infection than that of samples taken at beginning of infection (16.67%; 26/156), presented at Fig 4. The anti-FAdV antibody titers differs significantly at 99% confidence level ($p<0.01$) in eight flocks and at 95% confidence level ($p<0.05$) in one flock at beginning of infection and after recovery from infection (Table 4).

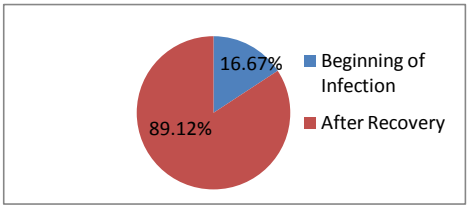


Figure 4: Pie chart showing Anti-FAdV antibody prevalence

Table 4: Anti-FAdV (Group 1) antibodies ELISA data in chicken serum (n=303)

Flock type	Status	Age (days)	No. of sample	Minimum/ Maximum Titer	Mean Titer ±SE	Number/ Percent seropositive	p value	Significance
B1	BE	9	14	211/2984	828±236	2/14.28	0.019	*
	AR	35	9	1130/2502	1516±128	9/100	p<0.05	
B2	BE	7	14	211/1285	493±76	1/7.14	p<0.01	**
	AR	32	15	1488/6688	3872±431	15/100		
B3	BE	11	14	139/721	474±44	0/0	p<0.01	**
	AR	32	17	1367/10597	7833±605	17/100		
BB1	BE	14	18	115/1256	399±81	2/11.11	p<0.01	**
	AR	56	15	522/2185	1246±172	8/53.33		
BB2	BE	9	18	215/1427	644±84	3/16.66	p<0.01	**
	AR	60	16	2624/10379	5457±650	16/100		
BB3	BE	8	18	168/2198	666±151	4/22.22	p<0.01	**
	AR	70	15	5548/14241	9699±832	15/100		
BB4	BE	14	15	26/1355	446±111	3/20	p<0.01	**
	AR	57	15	611/2160	1451±137	12/80		
BB5	BE	16	15	178/1524	739±121	5/33.33	p<0.01	**
	AR	57	15	791/9499	7417±634	14/93.33		
BB6	BE	16	15	151/2170	885±172	6/40	p<0.01	**
	AR	56	15	3650/13634	9003±820	15/100		
CL1	BE	35	15	141/393	218±16	0/0	0.915	NS
	AR	70	15	1/319	221±14	0/0	p>0.05	

NS = Not significant (p>0.05), * = Significant at 5% level of probability (p<0.05), ** = Significant at 1% level of probability (p<0.01)

BE= Beginning of infection, AR= After Recovery

4.4 Isolation of FAdV in SPF embryonated chicken eggs

Four days after being inoculated with the liver homogenate of the infected birds from the three suspected flocks, all of the embryos died. The inoculated embryos showed curling and dwarfing with enlarged greenish color liver in compared to the control embryos (plate 37, 38, 39).

4.5 Prevalence of FAdVs: FAdVs were investigated in fifty samples originated from different broiler flocks (*n=3*), broiler breeder flocks (*n=6*) and commercial layer flock (*n=1*) from Gazipur (*n=5*), Panchagarh (*n=4*) and Dinajpur (*n=1*) districts of Bangladesh. At flock level the prevalence of FAdVs was 90% (9/10) serologically. By using Rt-PCR, 37 out of 50 samples were confirmed to be positive for FAdVs, representing a 74% prevalence rate (Table 5, Fig.29). The samples were also tested by conventional PCR. By conventional PCR ~600 bp DNA fragment from loop-1 region of the *hexon* gene was amplified and a representative image is shown in Figure 5. There was complete agreement between the two tests' (realtime PCR and conventional PCR) results. Viruses were detected in samples of all broiler and broiler breeder flocks collected from Gazipur and Panchagarh districts of Bangladesh. Ages of virus positive chickens were within 7-15 days. All the liver homogenate samples of dead inoculated embryo also were positive both on conventional PCR and realtime PCR except control one. FAdV could not be detected in samples of commercial layer flock collected from Dinajpur district.

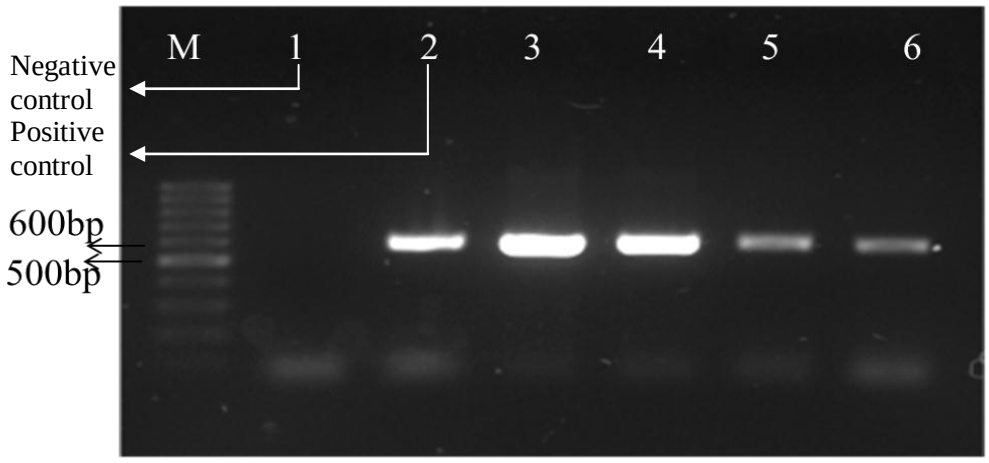


Figure 5: Amplification of loop 1 (L1) region of hexon gene of fowl adevoviruses. Lane M: 100bp DNA ladder (GeneRuler, Thermo Scientific), Lane1: Negative control, Lane 2: positive control, Lane 3-6: Test sample.

4.6 Circulating FAdV serotypes

Real time PCR kits with specificity for serotype 4 and serotype 8b were used. Besides, sequencing of loop1 region of hexon gene amplified by conventional PCR (Plate 40, 41) was done for serotyping of FAdV. Three serotypes of FAdV viz. FAdV-5 of species B, FAdV-8b of species E and FAdV-11 of species D were detected. FAdV-8b was found in seven flocks (B1-B3, BB1-BB4) out of nine positive flocks while FAdV-11 was found in two flocks (BB5, BB6) and FAdV-5 in one flock (BB2). FAdV-5 and FAdV-8b serotypes were detected from Gazipur district, while FAdV-8b and FAdV-11 serotypes were detected from samples of Panchagarh district. Serotype 8b was detected from samples of Atwary upazila while serotype 11 was detected from Tetulia upazila (Table 5) of Panchagarh district. Co-infection with serotype 8b and 5 was found in one broiler breeder flock (BB2) of Gazipur district. The detected serotypes of inoculated dead embryo were same as found in case of infected flocks (Fig.30-33).

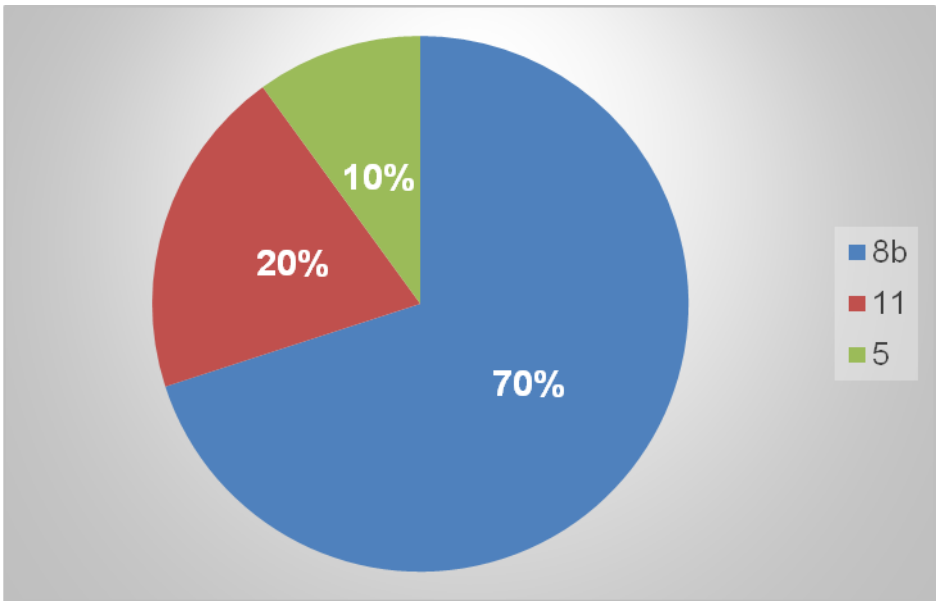


Figure 6: Pie chart showing the prevalence of different FAdV serotypes.

Table 5: Clinical signs, postmortem lesions and molecular detection of specific serotypes

Flock Type	Morbidity/ Mortality (%)	Clinical signs observed	Postmortem lesions	FAdV with serotype	GenBank accession no
Broiler (B1)	25/15	Anorexia, Depression, Reluctant to move, ruffled feathers, mortality	Enlarged hemorrhagic liver, swollen kidneys	5/5* S-8b	OP004931
Broiler (B2)	23/20	Drowsiness, anorexia, ruffled feathers, mortality	Tan colored liver, swollen spleen & kidneys	5/5 S-8b	OP502745
Broiler (B3)	30/16	Drowsiness, less body weight gain, anorexia, ruffled feathers, mortality	Hemorrhagic liver, hydrocardium, swollen kidneys	5/5 S-8b	-
Broiler breeder (BB1)	35/25	Drowsiness, lack of uniformity, mortality, ruffled feathers	Enlarged liver with hemorrhage, swollen kidneys & spleen	2/5 S-8b	OP454906
Broiler breeder (BB2)	40/20	Drowsiness, anorexia, ruffled feathers, mortality, reluctant to move	Hemorrhagic enlarged liver, congested pancreas, kidneys	5/5 S-5 3/5 S-8b	S-5 OP502746 S-8b- OP502747
Broiler breeder (BB3)	45/20	Anorexia, Drowsiness, Respiratory distress, ruffled feathers, mortality	Enlarged liver, fluid in pericardial sac, swollen kidneys with hemorrhage	5/5 S-8b	OP454908
Broiler breeder (BB4)	40/15	Drowsiness, Anorexia, depression, ruffled feathers, mortality	Hemorrhagic enlarged liver & hemorrhage at muscle & kidneys	5/5 S-8b	-
Broiler breeder (BB5)	35/12	Drowsiness, ruffled feather, sudden death, mortality	Pale enlarged liver, swollen kidneys	2/5 S-11	-
Broiler breeder (BB6)	65/30	Drowsiness, Anorexia, unable to move, ruffled, feathers, mortality	Pale enlarged liver, swollen kidneys	3/5 S-11	OP454907
Commercial layer (CL1)	10/5	Anorexia, Reluctant to move, mortality	Enlarged liver	0/5	

*Number of sample positive/Number of sample tested. S-8b: Serotype 8b, S-5: Serotype 5, S-11: Serotype 11; -: sequencing data not submitted for accession number but serotype was detected by sequences data on NCBI Blast, and serotype specific realtime PCR kit as mentioned in the methodology section.

4.7 Phylogenetic analysis

Evolutionary analyses of FAdVs were conducted in MEGA11 using the Neighbor-Joining method. The viruses were separated into three distinct clusters representing three different serotypes of FAdVs (Figure 7). In the tree Bangladeshi FAdV serotype 8b, serotype 11 and serotype 5 were clustered separately with other viruses. About 99.91% nucleotide identity was found among Bangladeshi FAdV serotype 8b. On the other hand 99.99% homology was found between two FAdV-5 serotypes. FAdV serotypes 5 of this study were found to have 100% homology at amino acid level. Similarly, 100% homology was also found in case of serotypes 8b. However, the distance between the serotype 5 and 8b was found 0.42%, between the serotypes 5 and 11 was 0.39% and between the serotypes 8b and 11 was 0.29%. Phylogenetic analysis revealed that the Bangladeshi FAdVs obtained from chicken has close identity with viruses from Asian (India, China, Indonesia, Korea), European (Austria, France, Hungary, Serbia) South American (Peru, Ecuador) and North American (Canada) countries. Bangladeshi FAdV serotype 8b has close relation with virus sequences from Peru (KX755572, 2015), Canada (EF685489, 2007), India (MH379248, 2018), Indonesia (MK692960, 2017). On the other hand serotype 11 was found to have close relation with virus reported from South Korea (HQ697595) in 2008. Besides, our serotype 11 have also clustered with viruses from Serbia (OM858815, 2021), Ecuador (MF161434, 2016) and so on. Additionally, Bangladeshi serotype 5 have clustered with viruses from China (OM836676, 2021), Hunagry (OK283055, 2019), Austria (OK283047, 2015) and France (OK283051, 2015) isolated since 2015.

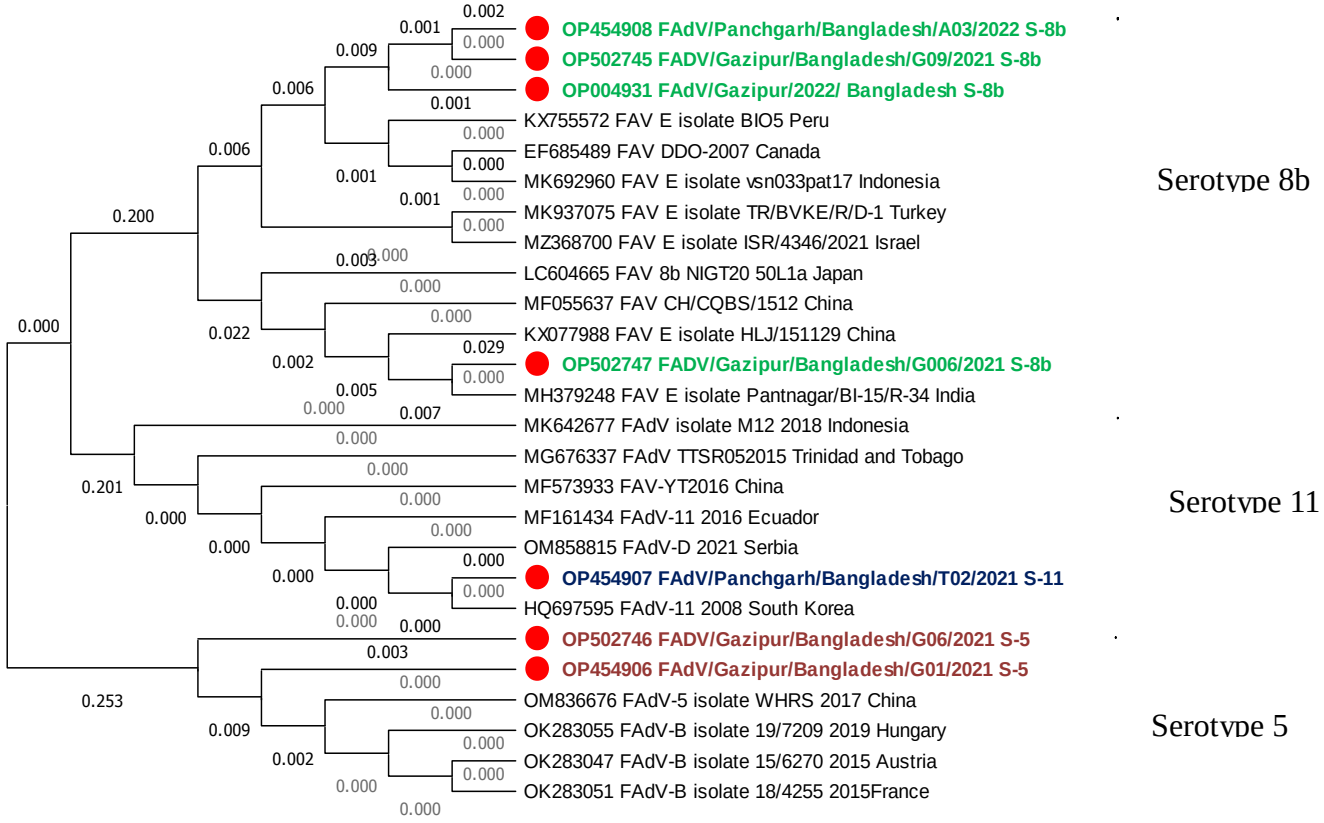


Figure 7: Phylogenetic analysis (Evolutionary relationships) of fowl adenoviruses. Evolutionary analyses were conducted in MEGA11. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Viruses sequenced in this study were marked with red circle. Different serotypes were different color. Green color: Fowl adenovirus serotype 8b. Blue: serotype 11, Dark red: serotype 5.

Plate 37: Isolation of Fadv using ECE, Control embryo and infected embryo (shows curling and dwarfing) after 4days of inoculation

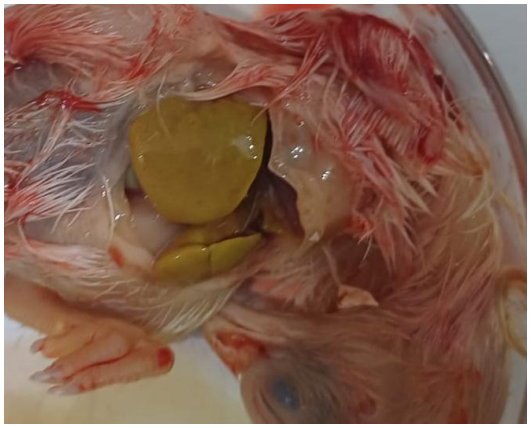


Plate 38: Inoculated embryo with enlarged, greenish liver

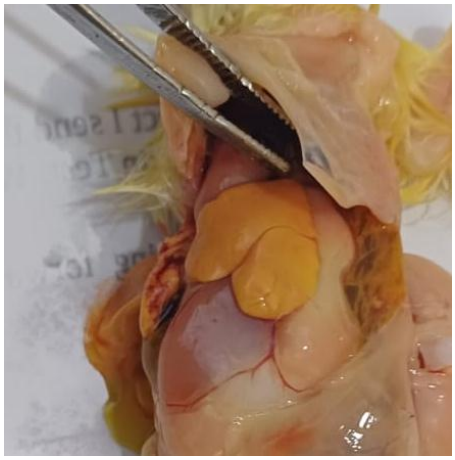
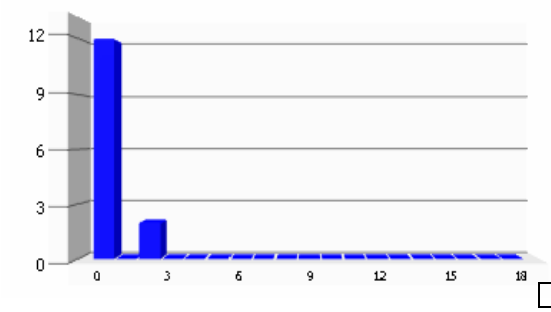


Plate 39: Control embryo with normal liver

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**9 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**06/06/2021**		
Mean Titer:	**828**	No. Samples:	**14**
Min-Max Titer:	**211 - 2984**	Neg/Sus/Pos:	**12/0/2**
GMT:	**574**		
%CV:	**107**		

[VI Index:] **7**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2078)**

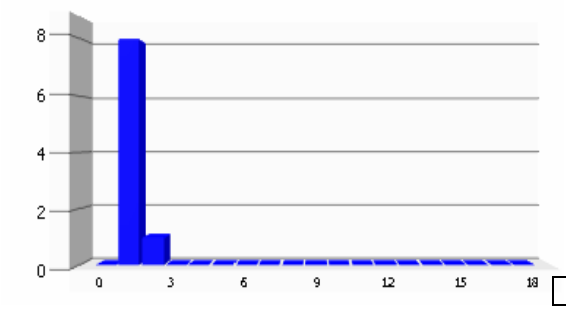
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.239				
-	B01	0.253				
+	C01	0.818				
+	D01	0.796				
01	G01	0.958	1.269	2984	2	[pos]
02	H01	0.397	0.269	542	0	[neg]
03	A02	0.434	0.335	690	0	[neg]
04	B02	0.320	0.132	248	0	[neg]
05	C02	0.310	0.114	211	0	[neg]
06	D02	0.480	0.417	877	0	[neg]
07	E02	0.440	0.346	714	0	[neg]
08	F02	0.416	0.303	617	0	[neg]
09	G02	0.422	0.314	642	0	[neg]
10	H02	0.392	0.260	522	0	[neg]
11	A03	0.320	0.132	248	0	[neg]
12	B03	0.901	1.168	2724	2	[pos]
13	C03	0.344	0.175	338	0	[neg]
14	D03	0.319	0.130	243	0	[neg]

Figure 8: F-B1. Relatively lower antibody titer at 10days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**35 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**01/07/2021**		
Mean Titer:	**1516**	No. Samples:	**9**
Min-Max Titer:	**1130 - 2502**	Neg/Sus/Pos:	**0/0/9**
GMT:	**1483**		
%CV:	**25**		

[VI Index:] **60**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1985)**

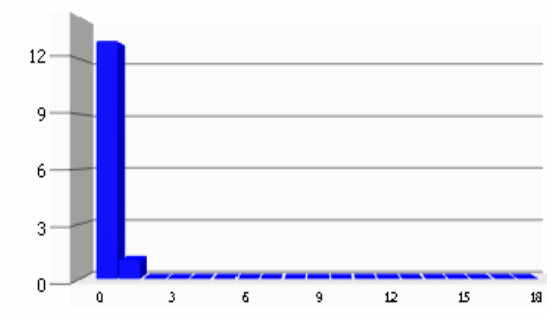
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.236				
-	B01	0.249				
+	C01	0.809				
+	D01	0.805				
01	G01	0.594	0.623	1364	1	[pos]
02	H01	0.599	0.632	1386	1	[pos]
03	A02	0.629	0.685	1514	1	[pos]
04	B02	0.612	0.655	1442	1	[pos]
05	C02	0.608	0.647	1422	1	[pos]
06	D02	0.853	1.081	2502	2	[pos]
07	E02	0.539	0.525	1130	1	[pos]
08	F02	0.609	0.649	1427	1	[pos]
09	G02	0.617	0.663	1461	1	[pos]

Figure 9: F-B1. Significantly higher antibody titer at 35days (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**7 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-2**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**10/06/2021**		
Mean Titer:	**493**	No. Samples:	**14**
Min-Max Titer:	**211 - 1285**	Neg/Sus/Pos:	**13/0/1**
GMT:	**433**		
%CV:	**57**		

[VI Index:] **8**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2078)**

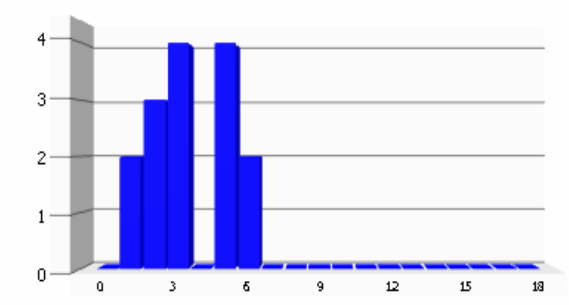
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.239				
-	B01	0.253				
+	C01	0.818				
+	D01	0.796				
01	E03	0.325	0.141	266	0	[neg]
02	F03	0.426	0.321	658	0	[neg]
03	G03	0.359	0.201	393	0	[neg]
04	H03	0.310	0.114	211	0	[neg]
05	A04	0.318	0.128	239	0	[neg]
06	B04	0.577	0.590	1285	1	[pos]
07	C04	0.428	0.324	665	0	[neg]
08	D04	0.380	0.239	476	0	[neg]
09	E04	0.330	0.150	285	0	[neg]
10	F04	0.404	0.282	571	0	[neg]
11	G04	0.413	0.298	606	0	[neg]
12	H04	0.360	0.203	397	0	[neg]
13	A05	0.317	0.127	237	0	[neg]
14	B05	0.415	0.301	613	0	[neg]

Figure 10: F-B2. Relatively lower antibody titer at 7days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**32 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-2**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**07/07/2021**		
Mean Titer:	**3872**	No. Samples:	**15**
Min-Max Titer:	**1488 - 6488**	Neg/Sus/Pos:	**0/0/15**
GMT:	**3515**		
%CV:	**43**		

[VI Index:] **90**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1985)**

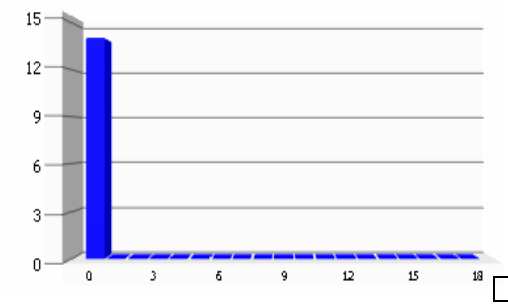
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.236				
-	B01	0.249				
+	C01	0.809				
+	D01	0.805				
01	H02	0.689	0.791	1774	1	[pos]
02	A03	1.431	2.105	5207	5	[pos]
03	B03	1.399	2.049	5055	5	[pos]
04	C03	1.400	2.050	5057	5	[pos]
05	D03	1.685	2.555	6444	6	[pos]
06	E03	1.120	1.554	3729	3	[pos]
07	F03	1.519	2.261	5633	5	[pos]
08	G03	0.848	1.073	2481	2	[pos]
09	H03	0.805	0.996	2286	2	[pos]
10	A04	1.016	1.370	3246	3	[pos]
11	B04	1.095	1.510	3613	3	[pos]
12	C04	1.694	2.571	6488	6	[pos]
13	D04	1.033	1.400	3325	3	[pos]
14	E04	0.623	0.674	1488	1	[pos]
15	F04	0.799	0.986	2261	2	[pos]

Figure 11: F-B2. Significantly higher antibody titer at 34days (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**11 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-3**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**20/06/2021**		
Mean Titer:	**474**	No. Samples:	**14**
Min-Max Titer:	**139 - 721**	Neg/Sus/Pos:	**14/0/0**
GMT:	**438**		
%CV:	**35**		

[VI Index:] **13**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2078)**

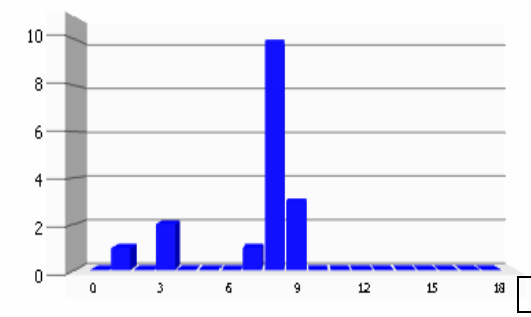
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.239				
-	B01	0.253				
+	C01	0.818				
+	D01	0.796				
01	C05	0.405	0.283	573	0	[neg]
02	D05	0.351	0.187	363	0	[neg]
03	E05	0.442	0.349	721	0	[neg]
04	F05	0.401	0.276	557	0	[neg]
05	G05	0.390	0.257	515	0	[neg]
06	H05	0.342	0.171	329	0	[neg]
07	A06	0.395	0.266	535	0	[neg]
08	B06	0.420	0.310	633	0	[neg]
09	C06	0.354	0.193	376	0	[neg]
10	D06	0.290	0.078	139	0	[neg]
11	E06	0.410	0.292	593	0	[neg]
12	F06	0.401	0.276	557	0	[neg]
13	G06	0.311	0.116	215	0	[neg]
14	H06	0.396	0.267	537	0	[neg]

Figure 12: F-B3. Relatively lower antibody titer at 12days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**32 Day(s)**
Type :	**Broilers**
Breed :	**Indian River**
Flock No :	**B-3**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**11/07/2021**		
Mean Titer:	**7833**	No. Samples:	**17**
Min-Max Titer:	**1367 - 10579**	Neg/Sus/Pos:	**0/0/17**
GMT:	**7161**		
%CV:	**32**		

[VI Index:] **244**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1985)**

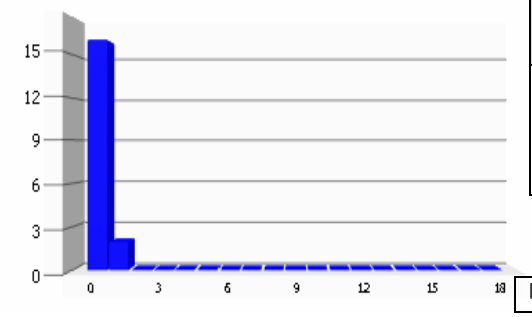
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.236				
-	B01	0.249				
+	C01	0.809				
+	D01	0.805				
01	G04	2.125	3.335	8638	8	[pos]
02	H04	2.092	3.276	8470	8	[pos]
03	A05	1.916	2.965	7590	7	[pos]
04	B05	2.115	3.317	8587	8	[pos]
05	C05	1.103	1.524	3650	3	[pos]
06	D05	2.139	3.360	8709	8	[pos]
07	E05	2.137	3.356	8698	8	[pos]
08	F05	0.595	0.624	1367	1	[pos]
09	G05	1.130	1.572	3777	3	[pos]
10	H05	2.096	3.283	8490	8	[pos]
11	A06	2.093	3.278	8476	8	[pos]
12	B06	2.415	3.849	10113	9	[pos]
13	C06	2.147	3.374	8749	8	[pos]
14	D06	2.108	3.305	8552	8	[pos]
15	E06	2.139	3.360	8709	8	[pos]
16	F06	2.396	3.815	10015	9	[pos]
17	G06	2.506	4.010	10579	9	[pos]

Figure 13: F-B3. Significantly higher antibody titer at 33days (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**14 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**04/12/2021**		
Mean Titer:	**399**	No. Samples:	**18**
Min-Max Titer:	**115 - 1256**	Neg/Sus/Pos:	**16/0/2**
GMT:	**308**		
%CV:	**86**		

[VI Index:] **4**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2078)**

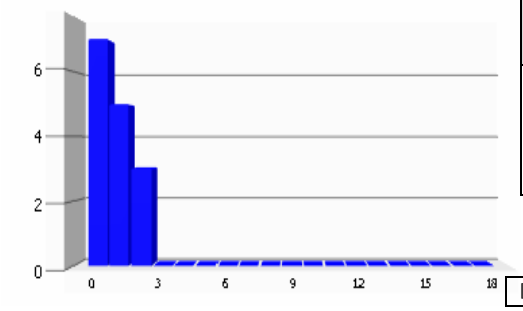
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.239				
-	B01	0.253				
+	C01	0.818				
+	D01	0.796				
01	A07	0.283	0.066	115	0	[neg]
02	B07	0.306	0.107	196	0	[neg]
03	C07	0.306	0.107	196	0	[neg]
04	D07	0.310	0.114	211	0	[neg]
05	E07	0.570	0.578	1256	1	[pos]
06	F07	0.313	0.119	221	0	[neg]
07	G07	0.332	0.153	291	0	[neg]
08	H07	0.310	0.114	211	0	[neg]
09	A08	0.320	0.132	248	0	[neg]
10	B08	0.417	0.305	622	0	[neg]
11	C08	0.311	0.116	215	0	[neg]
12	D08	0.560	0.560	1213	1	[pos]
13	E08	0.327	0.144	272	0	[neg]
14	F08	0.424	0.317	649	0	[neg]
15	G08	0.340	0.168	323	0	[neg]
16	H08	0.295	0.087	156	0	[neg]
17	A09	0.413	0.298	606	0	[neg]
18	B09	0.302	0.100	182	0	[neg]

Figure 14: F-BB1. Relatively lower antibody titer at 14days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**8 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**15/01/2022**		
Mean Titer:	**1245**	No. Samples:	**15**
Min-Max Titer:	**522 - 2185**	Neg/Sus/Pos:	**7/0/8**
GMT:	**1073**		
%CV:	**53**		

[VI Index:] **23**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1985)**

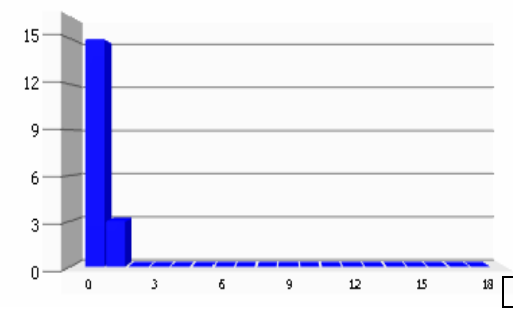
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.236				
-	B01	0.249				
+	C01	0.809				
+	D01	0.805				
01	H06	0.389	0.260	522	0	[neg]
02	A07	0.782	0.956	2185	2	[pos]
03	B07	0.395	0.270	544	0	[neg]
04	C07	0.602	0.637	1398	1	[pos]
05	D07	0.407	0.291	591	0	[neg]
06	E07	0.744	0.888	2015	2	[pos]
07	F07	0.407	0.291	591	0	[neg]
08	G07	0.726	0.857	1938	1	[pos]
09	H07	0.604	0.640	1405	1	[pos]
10	A08	0.616	0.662	1459	1	[pos]
11	B08	0.712	0.832	1876	1	[pos]
12	C08	0.780	0.952	2175	2	[pos]
13	D08	0.425	0.323	662	0	[neg]
14	E08	0.421	0.316	647	0	[neg]
15	F08	0.429	0.330	678	0	[neg]

Figure 15: F-BB1. Significantly higher antibody titer at 8wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**10 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-2**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**10/12/2021**		
Mean Titer:	**644**	No. Samples:	**18**
Min-Max Titer:	**215 - 1427**	Neg/Sus/Pos:	**15/0/3**
GMT:	**565**		
%CV:	**56**		

[VI Index:] **11**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2078)**

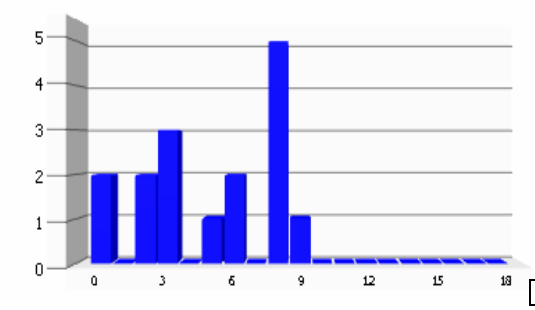
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.239				
-	B01	0.253				
+	C01	0.818				
+	D01	0.796				
01	C09	0.330	0.150	285	0	[neg]
02	D09	0.410	0.292	593	0	[neg]
03	E09	0.590	0.613	1340	1	[pos]
04	F09	0.428	0.324	665	0	[neg]
05	G09	0.405	0.283	573	0	[neg]
06	H09	0.404	0.282	571	0	[neg]
07	A10	0.311	0.116	215	0	[neg]
08	B10	0.404	0.282	571	0	[neg]
09	C10	0.394	0.264	531	0	[neg]
10	D10	0.427	0.323	662	0	[neg]
11	E10	0.610	0.649	1427	1	[pos]
12	F10	0.410	0.292	593	0	[neg]
13	G10	0.420	0.310	633	0	[neg]
14	H10	0.396	0.267	537	0	[neg]
15	A11	0.389	0.255	511	0	[neg]
16	B11	0.585	0.604	1319	1	[pos]
17	C11	0.323	0.137	258	0	[neg]
18	D11	0.340	0.168	323	0	[neg]

Figure 16: F-BB2. Relatively lower antibody titer at 10days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**9 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-2**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**30/01/2022**		
Mean Titer:	**5457**	No. Samples:	**16**
Min-Max Titer:	**1 - 10379**	Neg/Sus/Pos:	**2/0/14**
GMT:	**1927**		
%CV:	**59**		

[VI Index:] **92**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1985)**

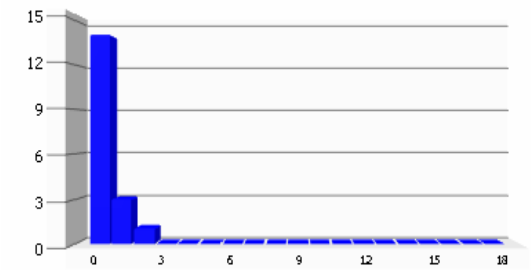
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.236				
-	B01	0.249				
+	C01	0.809				
+	D01	0.805				
01	G08	1.611	2.424	6081	6	[pos]
02	H08	0.091	0.000	1	0	[neg]
03	A09	1.111	1.539	3689	3	[pos]
04	B09	2.100	3.291	8513	8	[pos]
05	C09	0.924	1.207	2824	2	[pos]
06	D09	2.467	3.941	10379	9	[pos]
07	E09	0.880	1.129	2624	2	[pos]
08	F09	1.124	1.562	3750	3	[pos]
09	G09	2.100	3.291	8513	8	[pos]
10	H09	1.099	1.517	3631	3	[pos]
11	A10	2.107	3.303	8547	8	[pos]
12	B10	1.601	2.407	6034	6	[pos]
13	C10	0.090	0.000	1	0	[neg]
14	D10	2.120	3.326	8612	8	[pos]
15	E10	1.506	2.238	5570	5	[pos]
16	F10	2.108	3.305	8552	8	[pos]

Figure 17: F-BB2. Significantly higher antibody titer at 9wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**8 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-3**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**10/06/2022**		
Mean Titer:	**666**	No. Samples:	**18**
Min-Max Titer:	**168 - 2198**	Neg/Sus/Pos:	**14/0/4**
GMT:	**491**		
%CV:	**86**		

[VI Index:] **7**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1926)**

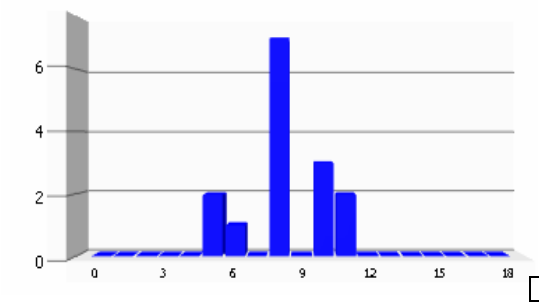
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.237				
-	B01	0.243				
+	C01	0.819				
+	D01	0.804				
01	G01	0.294	0.094	170	0	[neg]
02	H01	0.293	0.093	168	0	[neg]
03	A02	0.387	0.257	515	0	[neg]
04	B02	0.607	0.642	1410	1	[pos]
05	C02	0.393	0.268	539	0	[neg]
06	D02	0.789	0.961	2198	2	[pos]
07	E02	0.340	0.175	338	0	[neg]
08	F02	0.309	0.121	225	0	[neg]
09	G02	0.616	0.658	1449	1	[pos]
10	H02	0.389	0.261	524	0	[neg]
11	A03	0.616	0.658	1449	1	[pos]
12	B03	0.303	0.110	203	0	[neg]
13	C03	0.440	0.350	724	0	[neg]
14	D03	0.319	0.138	260	0	[neg]
15	E03	0.412	0.301	613	0	[neg]
16	F03	0.319	0.138	260	0	[neg]
17	G03	0.348	0.189	367	0	[neg]
18	H03	0.406	0.290	588	0	[neg]

Figure 18: F-BB3. Relatively lower antibody titer at 8days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**10 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-3**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**01/08/2022**		
Mean Titer:	**9699**	No. Samples:	**15**
Min-Max Titer:	**5548 - 14241**	Neg/Sus/Pos:	**0/0/15**
GMT:	**9240**		
%CV:	**32**		

[VI Index:] **303**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2127)**

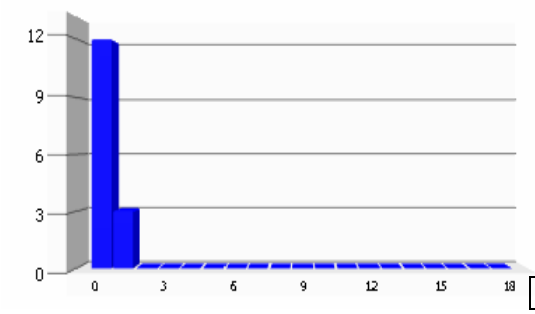
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.235				
-	B01	0.248				
+	C01	0.814				
+	D01	0.805				
01	G01	1.508	2.230	5548	5	[pos]
02	H01	2.055	3.193	8234	8	[pos]
03	A02	3.132	5.089	13750	10	[pos]
04	B02	2.072	3.223	8319	8	[pos]
05	C02	1.740	2.638	6674	6	[pos]
06	D02	2.138	3.339	8649	8	[pos]
07	E02	2.062	3.205	8268	8	[pos]
08	F02	2.137	3.337	8644	8	[pos]
09	G02	1.517	2.246	5592	5	[pos]
10	H02	2.088	3.251	8399	8	[pos]
11	A03	3.226	5.254	14241	11	[pos]
12	B03	2.970	4.804	12905	10	[pos]
13	C03	3.190	5.191	14053	11	[pos]
14	D03	3.112	5.054	13646	10	[pos]
15	E03	2.121	3.309	8564	8	[pos]

Figure 19: F-BB3. Significantly higher antibody titer at 10wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**14 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-4**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**15/06/2022**		
Mean Titer:	**446**	No. Samples:	**15**
Min-Max Titer:	**26 - 1355**	Neg/Sus/Pos:	**12/0/3**
GMT:	**278**		
%CV:	**93**		

[VI Index:] **4**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1926)**

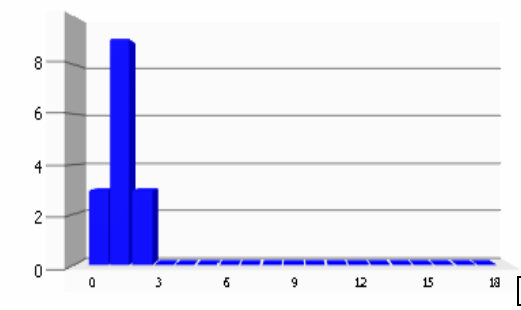
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.237				
-	B01	0.243				
+	C01	0.819				
+	D01	0.804				
01	A04	0.255	0.026	41	0	[neg]
02	B04	0.310	0.122	227	0	[neg]
03	C04	0.250	0.017	26	0	[neg]
04	D04	0.594	0.619	1355	1	[pos]
05	E04	0.323	0.145	274	0	[neg]
06	F04	0.299	0.103	188	0	[neg]
07	G04	0.324	0.147	279	0	[neg]
08	H04	0.292	0.091	164	0	[neg]
09	A05	0.528	0.504	1081	1	[pos]
10	B05	0.313	0.128	239	0	[neg]
11	C05	0.402	0.283	573	0	[neg]
12	D05	0.339	0.173	333	0	[neg]
13	E05	0.537	0.520	1118	1	[pos]
14	F05	0.296	0.098	178	0	[neg]
15	G05	0.413	0.303	617	0	[neg]

Figure 20: F-BB4. Relatively lower antibody titer at 14days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**8 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-4**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**28/07/2022**		
Mean Titer:	**1451**	No. Samples:	**15**
Min-Max Titer:	**611 - 2160**	Neg/Sus/Pos:	**3/0/12**
GMT:	**1349**		
%CV:	**35**		

[VI Index:] **41**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2127)**

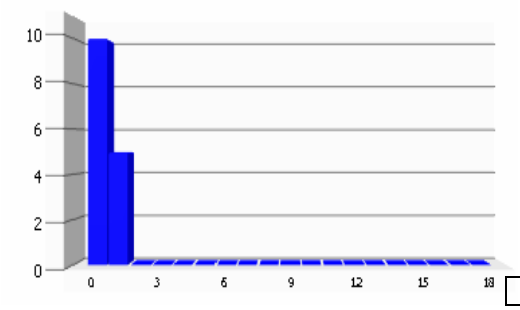
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.235				
-	B01	0.248				
+	C01	0.814				
+	D01	0.805				
01	F03	0.779	0.946	2160	2	[pos]
02	G03	0.647	0.714	1585	1	[pos]
03	H03	0.604	0.638	1401	1	[pos]
04	A04	0.611	0.651	1432	1	[pos]
05	B04	0.595	0.622	1362	1	[pos]
06	C04	0.634	0.691	1529	1	[pos]
07	D04	0.731	0.862	1950	1	[pos]
08	E04	0.422	0.318	651	0	[neg]
09	F04	0.771	0.932	2125	2	[pos]
10	G04	0.425	0.323	662	0	[neg]
11	H04	0.622	0.670	1478	1	[pos]
12	A05	0.412	0.300	611	0	[neg]
13	B05	0.773	0.936	2135	2	[pos]
14	C05	0.603	0.636	1396	1	[pos]
15	D05	0.580	0.596	1299	1	[pos]

Figure 21: F-BB4. Significantly higher antibody titer at 9wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**16 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-5**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**12/02/2021**		
Mean Titer:	**739**	No. Samples:	**15**
Min-Max Titer:	**178 - 1524**	Neg/Sus/Pos:	**10/0/5**
GMT:	**593**		
%CV:	**61**		

[VI Index:] **12**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1926)**

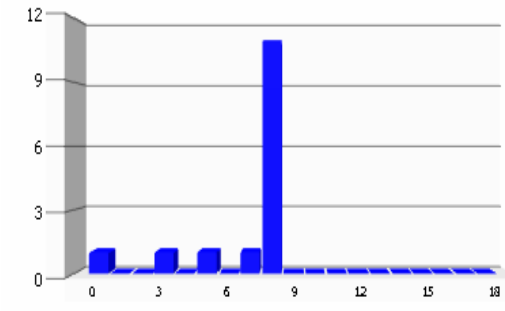
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.237				
-	B01	0.243				
+	C01	0.819				
+	D01	0.804				
01	H05	0.296	0.098	178	0	[neg]
02	A06	0.299	0.103	188	0	[neg]
03	B06	0.552	0.546	1180	1	[pos]
04	C06	0.340	0.175	338	0	[neg]
05	D06	0.408	0.294	597	0	[neg]
06	E06	0.634	0.689	1524	1	[pos]
07	F06	0.296	0.098	178	0	[neg]
08	G06	0.405	0.289	586	0	[neg]
09	H06	0.409	0.296	602	0	[neg]
10	A07	0.574	0.584	1271	1	[pos]
11	B07	0.416	0.308	629	0	[neg]
12	C07	0.606	0.640	1405	1	[pos]
13	D07	0.427	0.327	671	0	[neg]
14	E07	0.544	0.532	1147	1	[pos]
15	F07	0.408	0.294	597	0	[neg]

Figure 22: F-BB5. Relatively lower antibody titer at 17days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**8 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-5**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**25/03/2021**		
Mean Titer:	**7417**	No. Samples:	**15**
Min-Max Titer:	**719 - 9499**	Neg/Sus/Pos:	**1/0/14**
GMT:	**6572**		
%CV:	**32**		

[VI Index:] **231**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2127)**

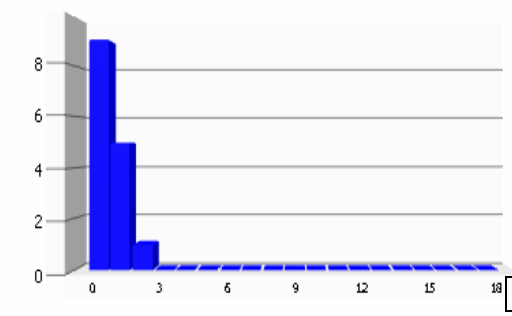
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.235				
-	B01	0.248				
+	C01	0.814				
+	D01	0.805				
01	E05	1.436	2.103	5201	5	[pos]
02	F05	2.095	3.263	8433	8	[pos]
03	G05	2.130	3.325	8609	8	[pos]
04	H05	2.095	3.263	8433	8	[pos]
05	A06	1.920	2.955	7562	7	[pos]
06	B06	2.104	3.279	8478	8	[pos]
07	C06	2.109	3.288	8504	8	[pos]
08	D06	2.307	3.636	9499	8	[pos]
09	E06	0.439	0.348	719	0	[neg]
10	F06	2.099	3.270	8453	8	[pos]
11	G06	2.106	3.283	8490	8	[pos]
12	H06	2.089	3.253	8405	8	[pos]
13	A07	2.085	3.246	8385	8	[pos]
14	B07	1.103	1.517	3631	3	[pos]
15	C07	2.101	3.274	8464	8	[pos]

Figure 23: F-BB5. Significantly higher antibody titer at 9wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**16 Day(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-6**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**15/02/2021**		
Mean Titer:	**885**	No. Samples:	**15**
Min-Max Titer:	**151 - 2170**	Neg/Sus/Pos:	**9/0/6**
GMT:	**623**		
%CV:	**76**		

[VI Index:] **11**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1926)**

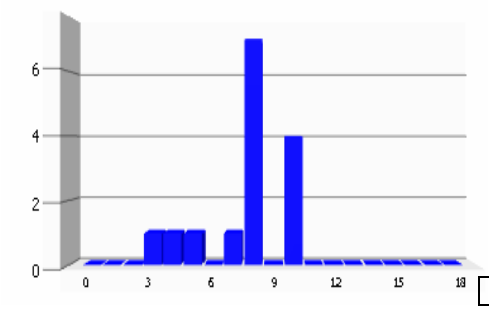
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.237				
-	B01	0.243				
+	C01	0.819				
+	D01	0.804				
01	G07	0.645	0.709	1573	1	[pos]
02	H07	0.305	0.114	211	0	[neg]
03	A08	0.321	0.142	268	0	[neg]
04	B08	0.637	0.695	1539	1	[pos]
05	C08	0.415	0.306	624	0	[neg]
06	D08	0.405	0.289	586	0	[neg]
07	E08	0.621	0.667	1471	1	[pos]
08	F08	0.412	0.301	613	0	[neg]
09	G08	0.621	0.667	1471	1	[pos]
10	H08	0.292	0.091	164	0	[neg]
11	A09	0.315	0.131	245	0	[neg]
12	B09	0.652	0.721	1602	1	[pos]
13	C09	0.783	0.950	2170	2	[pos]
14	D09	0.406	0.290	588	0	[neg]
15	E09	0.288	0.084	151	0	[neg]

Figure 24: F-BB6. Relatively lower antibody titer at 16days (beginning of infection)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**8 Week(s)**
Type :	**Broiler Breeder**
Breed :	**Indian River**
Flock No :	**BB-6**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**29/03/2021**		
Mean Titer:	**9003**	No. Samples:	**15**
Min-Max Titer:	**3650 - 13634**	Neg/Sus/Pos:	**0/0/15**
GMT:	**8383**		
%CV:	**37**		

[VI Index:] **243**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2127)**

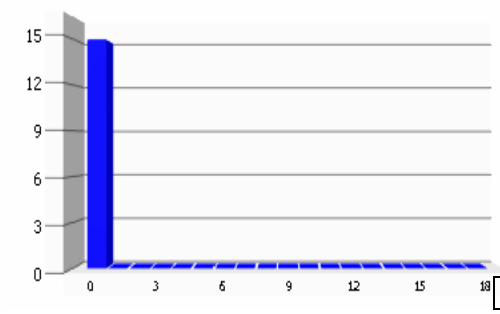
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.235				
-	B01	0.248				
+	C01	0.814				
+	D01	0.805				
01	D07	1.107	1.524	3650	3	[pos]
02	E07	2.144	3.349	8678	8	[pos]
03	F07	2.108	3.286	8498	8	[pos]
04	G07	2.127	3.320	8595	8	[pos]
05	H07	1.504	2.223	5529	5	[pos]
06	A08	1.912	2.941	7522	7	[pos]
07	B08	2.211	3.467	9015	8	[pos]
08	C08	3.109	5.048	13628	10	[pos]
09	D08	3.104	5.040	13604	10	[pos]
10	E08	2.021	3.133	8064	8	[pos]
11	F08	1.210	1.705	4129	4	[pos]
12	G08	2.111	3.291	8513	8	[pos]
13	H08	2.091	3.256	8413	8	[pos]
14	A09	3.110	5.050	13634	10	[pos]
15	B09	3.100	5.033	13583	10	[pos]

Figure 25: F-BB6. Significantly higher antibody titer at 8wks (after recovery)

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**35 Day(s)**
Type :	**Commercial Layer**
Breed :	**Indian River**
Flock No :	**CL-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**03/11/2020**		
Mean Titer:	**218**	No. Samples:	**15**
Min-Max Titer:	**141 - 393**	Neg/Sus/Pos:	**15/0/0**
GMT:	**210**		
%CV:	**30**		

[VI Index:] **7**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (1926)**

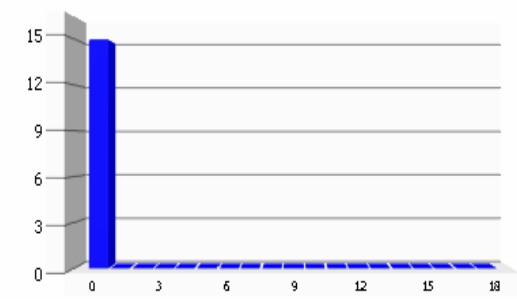
[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.237				
-	B01	0.243				
+	C01	0.819				
+	D01	0.804				
01	F09	0.355	0.201	393	0	[neg]
02	G09	0.310	0.122	227	0	[neg]
03	H09	0.299	0.103	188	0	[neg]
04	A10	0.308	0.119	221	0	[neg]
05	B10	0.303	0.110	203	0	[neg]
06	C10	0.289	0.086	155	0	[neg]
07	D10	0.312	0.126	235	0	[neg]
08	E10	0.306	0.115	213	0	[neg]
09	F10	0.308	0.119	221	0	[neg]
10	G10	0.297	0.100	182	0	[neg]
11	H10	0.293	0.093	168	0	[neg]
12	A11	0.288	0.084	151	0	[neg]
13	B11	0.285	0.079	141	0	[neg]
14	C11	0.319	0.138	260	0	[neg]
15	D11	0.334	0.164	314	0	[neg]

Figure 26: F-CL1. All the samples are negative at beginning of infection

Report: Histogram/Blockdiagraam

Lab code :	**TEST**
Farm Name :	
Company :	
Age :	**10 Week(s)**
Type :	**Commercial Layer**
Breed :	**Hy-Line**
Flock No :	**CL-1**
Shed No. :	
Reason :	**SCREENING**



Assay:	**FAV1**	Lot number:	**CH1757**
Bleeding date:	**07/12/2020**		
Mean Titer:	**169**	No. Samples:	**15**
Min-Max Titer:	**1 - 319**	Neg/Sus/Pos:	**15/0/0**
GMT:	**71**		
%CV:	**59**		

[VI Index:] **2**

Titer Range Ref.Controls	**CR100 (RF16) (1500-5000)**
Meantiter Ref.Controls	**CR100 (RF16) (2127)**

[Positive Cuttoff S/P:] >= **0.5**

Sample ID	Well	Raw OD	[S/P	[Titer]	Titer Group	Result
-	A01	0.235				
-	B01	0.248				
+	C01	0.814				
+	D01	0.805				
01	C09	0.324	0.145	274	0	[neg]
02	D09	0.336	0.166	319	0	[neg]
03	E09	0.288	0.082	147	0	[neg]
04	F09	0.324	0.145	274	0	[neg]
05	G09	0.100	0.000	1	0	[neg]
06	H09	0.299	0.101	184	0	[neg]
07	A10	0.306	0.114	211	0	[neg]
08	B10	0.301	0.105	192	0	[neg]
09	C10	0.189	0.000	1	0	[neg]
10	D10	0.312	0.124	231	0	[neg]
11	E10	0.000	0.000	1	0	[neg]
12	F10	0.307	0.115	213	0	[neg]
13	G10	0.298	0.099	180	0	[neg]
14	H10	0.292	0.089	160	0	[neg]
15	A11	0.292	0.089	160	0	[neg]

Figure 27: F-CL1. All the samples are also negative at 10wks (after recovery)

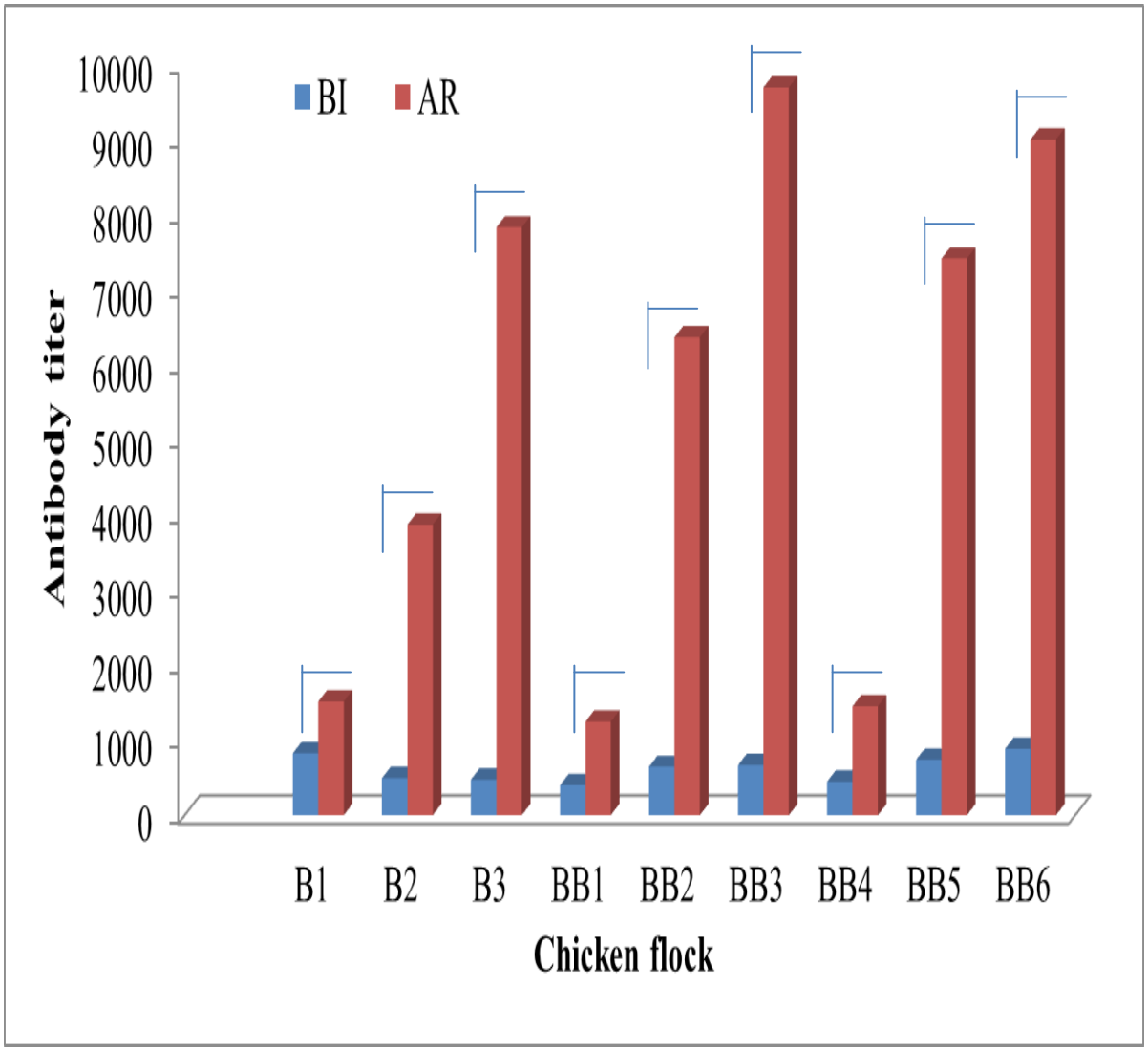


Figure 28: Mean antibody titer at the beginning of infection (BI) & after recovery (AR)

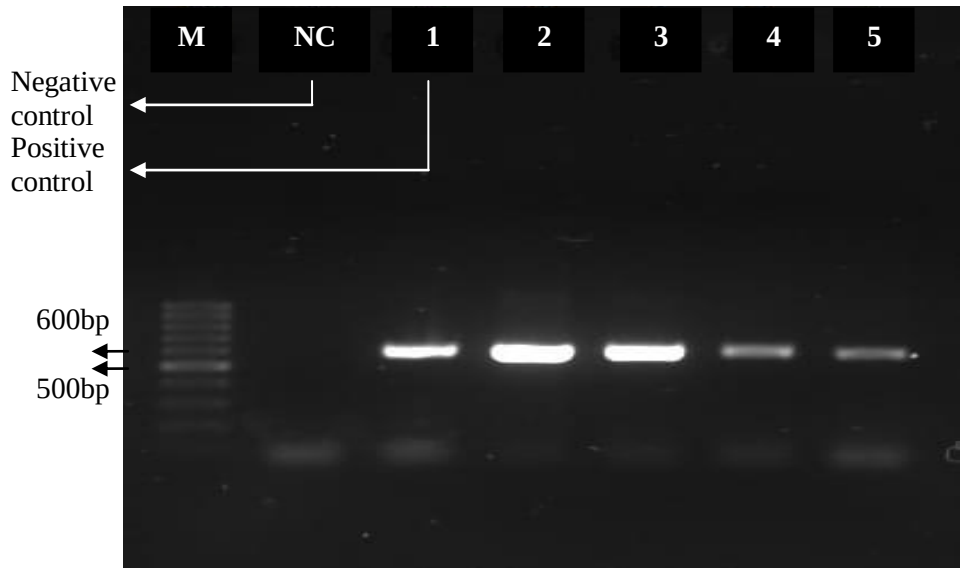


Plate 40: Results of conventional PCR with negative control, samples and DNA ladder (Lane M: 100bp DNA ladder (Lane NC: Negative control. Lane 1: positive control, Lane 2-5: positive samples of infected liver).

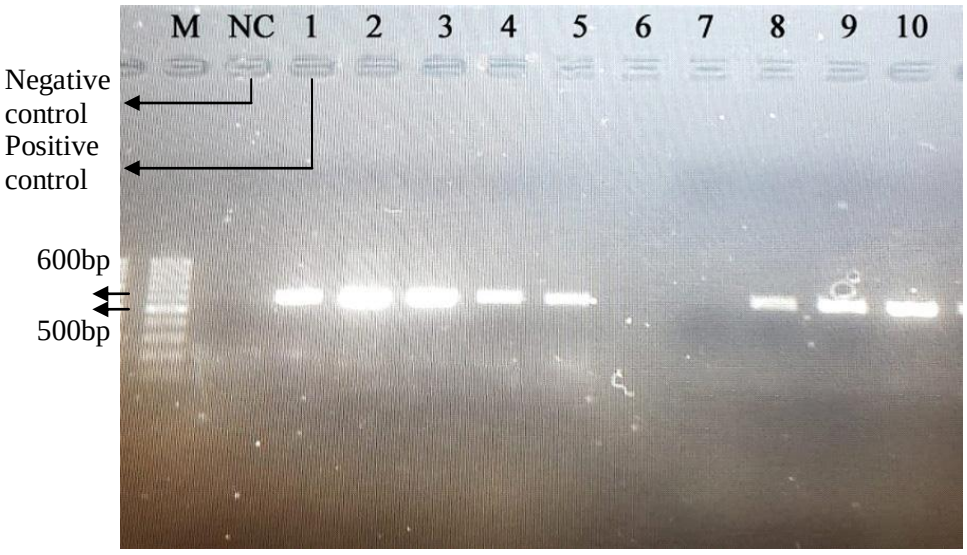


Plate 41: Results of conventional PCR with negative control, dead bird's samples, inoculated samples and DNA ladder. Lane M: 100bp DNA ladder (GeneRuler, Thermo Scientific), Lane NC: Negative control. Lane 1: positive control, Lane 2-5: positive sample, Lane 6-7: negative sample, Lane:8-10 positive inoculated samples.

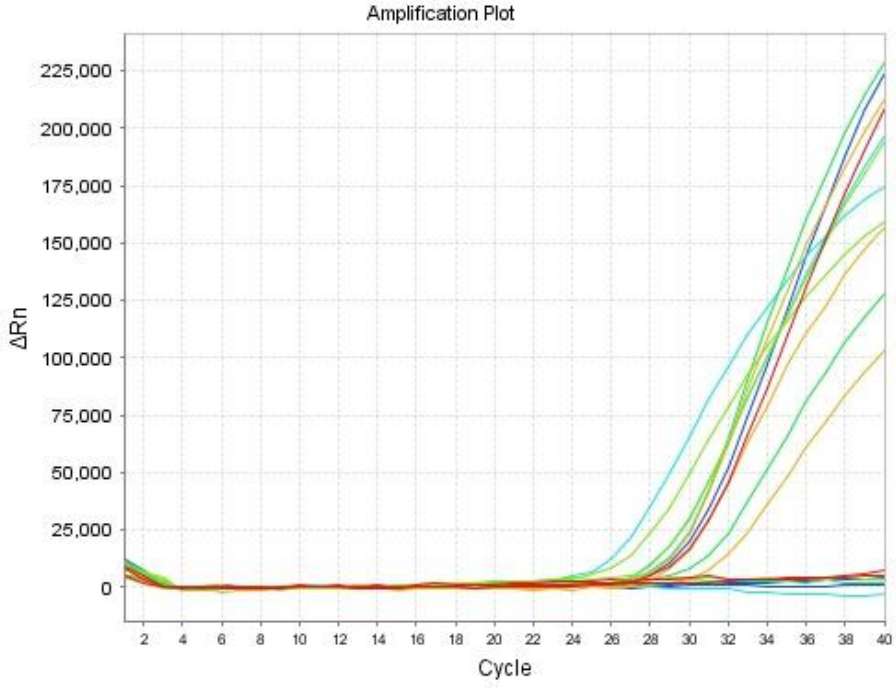


Figure 29: Amplification curve of multiple samples and serotypes on real time PCR

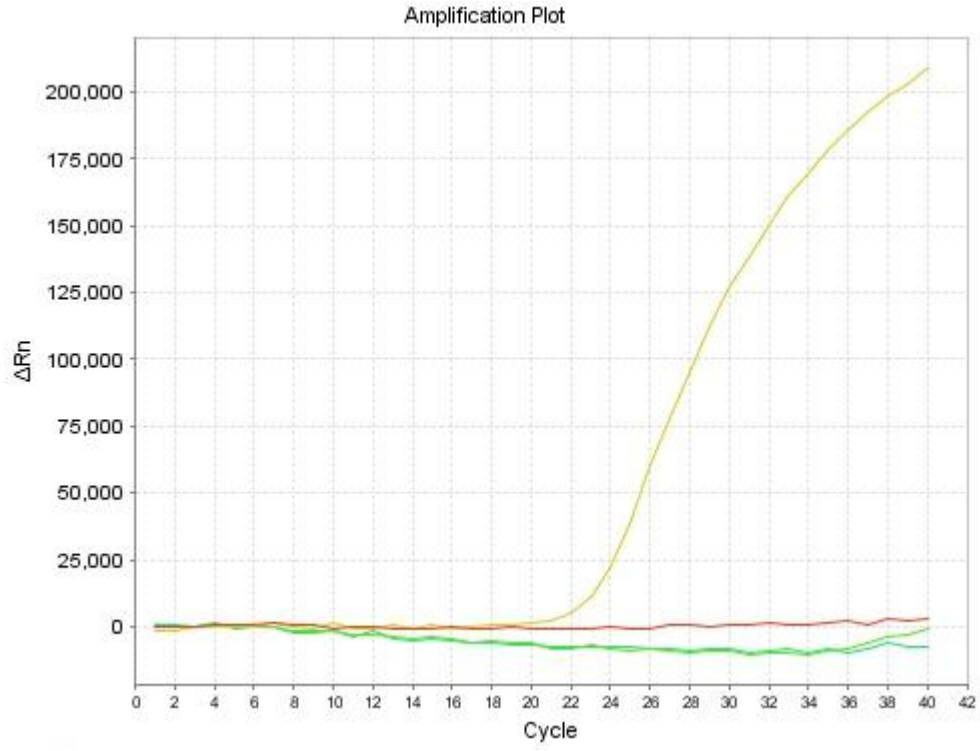


Figure 30: Amplification curve of serotype 8b on real time PCR (F-B1, dead bird)

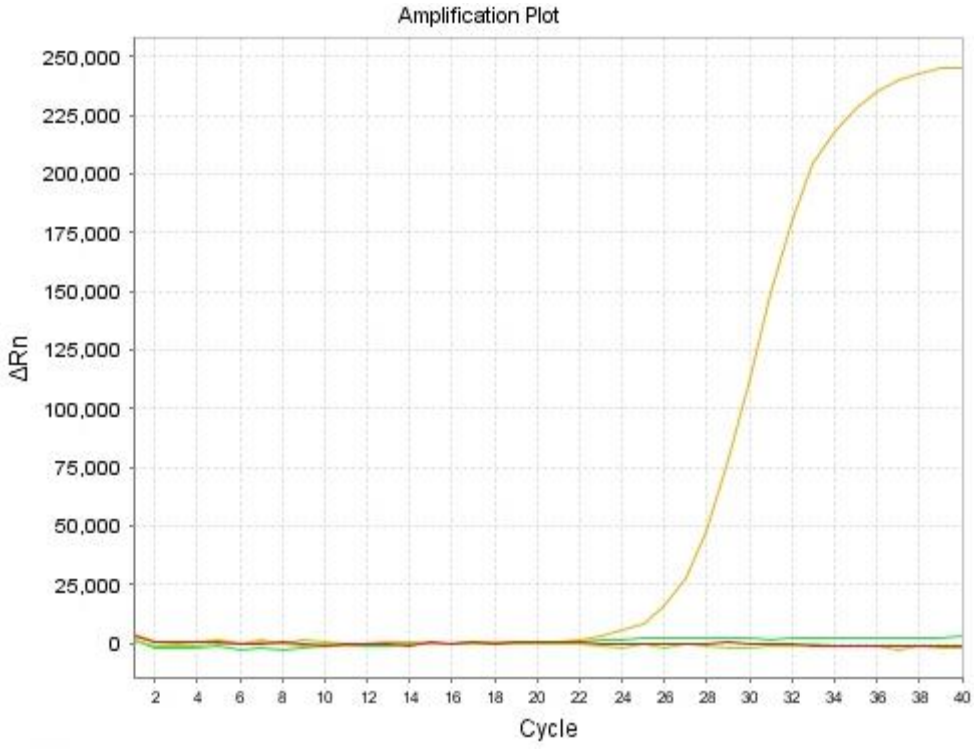


Figure 31: Amplification curve of serotype 8b on real time PCR. (F-B1, inoculated embryo)

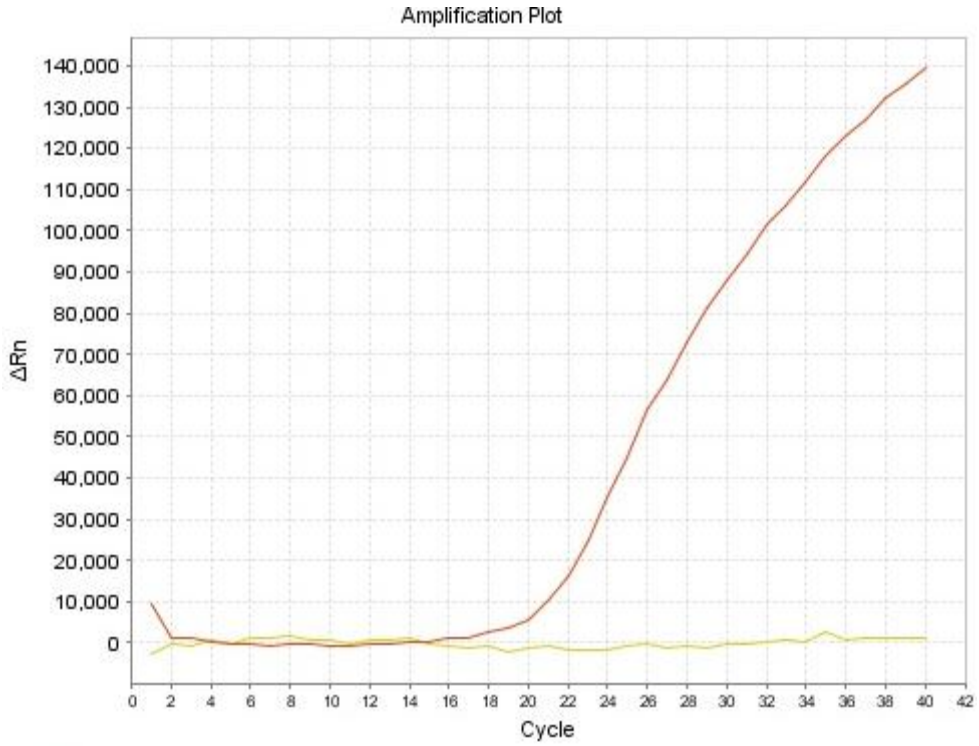


Figure 32: Amplification curve of serotype 8b on real time PCR (F-BB3 infected dead birds)

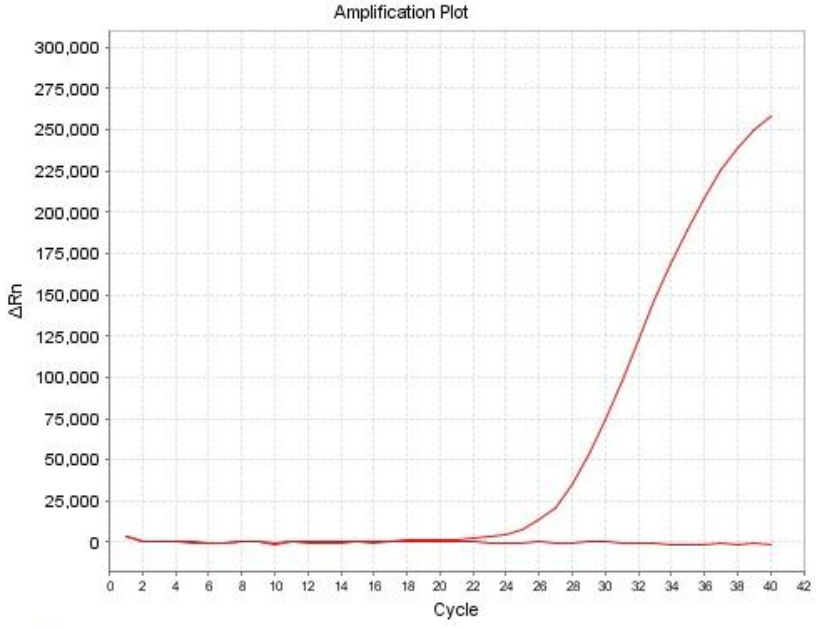


Figure 33: Amplification curve of serotype 8b on real time PCR (F-BB3 inoculated embryo)

CHAPTER-5

DISCUSSION

Three types of chicken- namely (i) commercial Broiler, (ii) Broiler breeder and (iii) Commercial layer chicken were investigated. It is to be noted that usually commercial broilers were reared in open-sided house with high density of birds. Generally crumble type feed was provided at starter stage and pellets at grower and finisher stage. Biosecurity measures in broiler farms was limited and farmed very close to locality, highway, and other commercial poultry operations. On the other hand, broiler breeders were reared in controlled sheds and crumble feed was given from starter stage to whole production period. The farm area was isolated from highway and other poultry operations. All in and all out system was usually followed with comprehensive biosecurity measures. In case of commercial layer, birds were reared in cage with crumble feed as starter and mash feed in production period. The farm area was usually isolated from highway and other poultry operations. Moderate biosecurity measures were ensured. The flocks examined in this study were collected from different three regions of Bangladesh. Two serotypes causing IBH mostly were detected from Panchagarh as more types of birds (GP, PS) were imported there; on the other hand only one specific serotype was detected from Gazipur. In Dinajpur region (in case of commercial layer) the incidence of IBH was not found most probably due to different case system rearing management, isolated area and environment. The present study showed that FAdV infections had affected various ages of chickens with different clinical signs. Almost all the flocks exhibited clinical signs of anorexia, drowsiness, ruffled feathers, lower body weight, lack of uniformity and increased mortality. However, clinical signs gradually declined after 2-3 weeks of first appearance. The virus might be transmitted by horizontally and/or vertically. The route of virus transmission cannot be determined from this study whether the transmission occurs vertical or horizontal. Mortality during IBH outbreaks may peaks within 3–4 days of clinical infection and can reach 10% and occasionally be as high as 30%

(Schachner *et al.,* 2018; Nakamura *et al.,* 2011; Choi *et al.,* 2012; Zhao *et al.,* 2015). The findings of 15-25% mortality, clinical signs and postmortem lesions (Table 5) are in concordance with the findings of above studies. Moreover, IBH induced 100% mortality in commercial broiler chickens was reported from Malaysia in 2005 (Hair-Bejo, 2005). Lower level Anti-FAdV antibody was detected at the beginning of infection and the ages of birds were within 7-16 days (Table 4). It indicates that this antibody was not due to exposure of the sampled birds to FAdVs. This antibody might be derived from mother vertically. Exposure of parent birds to circulating field FAdVs or use of FAdV vaccine either in combination with other vaccine or as single vaccine in parents might induced antibody in them and later passed to the chicks through egg yolk. Bangladesh does not formally use the FAdV vaccination which indicates field exposure to the parents at production may transfer MDA to their off-springs. It was common to find that antibody titers were initially low at the onset of infection but increased significantly ($p<0.05$) following recovery (Table-4). Because exposed birds might develop high level of antibody after exposure. It was not possible to determine whether the antibody is against serotype 8b, 11, or 5 due to the fact that an indirect ELISA kit was employed which detects antibodies to Group-1 FAdVs and does not different specific serotypes. In the serum samples taken from layer flocks in the Dinajpur district antibodies were not detected, even after they had supposedly recovered from an infection. This result agreed with the results of the virus detection. Thus, additional causes might be responsible for the clinical signs, morbidity, and mortality seen in this commercial layer flock. FAdV was also isolated from liver homogenates in SPF embryonated chicken eggs in the present study. The infected embryos exhibited curling and dwarfing in comparison to the control embryo, and their embryos were hemorrhagic with enlarged livers and diffuse greenish coloration. These findings also matched with previous IBH reports (Abghour *et al.*, 2019). The liver homogenates harvested from inoculated ECEs were also subjected to molecular detection of FAdVs. Both the samples (directly collected from infected dead birds and inoculated ECEs) have showed

positive results of same serotypes by PCR. This scenario indicates the proper propagation of virus in ECE and infection of flocks by a specific serotype.

The serotype 8b of genotype E was found as the most prevalent FAdV in studied chicken population in Bangladesh. Circulating other two serotypes included FAdV 5 (genotype B) and 11 (genotype D). It is reported that during last ten years the most reported IBH-induced serotypes were belongs to species D and E (Steer *et al.,* 2011). Several other studies also reported that IBH outbreaks have been confirmed in different countries and serotypes 2, 4, 8a, 8b and 11 was the most frequent serotypes as a causal agents (Schachner *et al.,* 2016; McFerran and Smyth, 2000; Wibowo *et al.,* 2019). Serotype 11 of species E was found to cause higher morbidity and mortality in the present study. This finding is also in line with the reports of (Matos *et al.,* 2016) who reported that birds infected with FAdV serotype 8b and 11 showed more severe clinical signs and mortalities than those inoculated with FAdV serotypes 2, 7 and 8a. Though clinical signs like anorexia, reluctant to move, postmortem lesions viz. enlarged liver and mortality (5%) were found in commercial layer flock, we could not detect FAdV from this group of chicken. Even they were serologically negative at beginning of clinical sign or infection and after recovery from infection. These findings suggest that other pathogen may be responsible for inducing those pathological conditions. Co-infection with FAdV serotype 8b and 5 was found in flock BB2. We could not detect serotype 5 from samples of other flocks. So, there is no chance of contamination with serotype 5 from other flocks. Additionally, there was little chance of laboratory contamination with serotype 8b. Because of the samples of flock BB2 were collected and tested in December 2021. There were no other samples collected and tested in the laboratory at nearby dates of December 2021. Hence, this co-infection was not due to contamination and laboratory originated. In a recent study (Liu *et al.,* 2021) showed co-infection with FAdV serotype 4 and 8b. They reported similar clinical symptoms, mortality rates and degree of tissue lesions in co-infected birds as in single infection with serotype 4. However, they found that co-infection with FAdV-4 and FAdV-8a suppresses the

replication and proliferation of FAdV-4 but enhances the replication and proliferation of FAdV-8a in chicken liver. These means co-infection may worsen the clinical conditions of chicken.

Phylogenetically Bangladeshi FAdVs were distinctly branched in to three clusters of serotypes 8b, 5 and 11 with viruses from Asian, European, North American and South American countries of the world (Figure 7). Hence, it is assumed that multiple introduction of FAdV in Bangladesh may be happened. High homology among the serotypes may also suggest spread of viruses within the country. One FAdV-8 serotype of this study has been linked to an Indian virus and the virus was detected in the sample of broiler breeder flock BB2. In Bangladesh' poultry industry was formed by the importation of chickens, eggs, feed materials and utensils, from several countries where FAdVs were recorded. The introduction of FAdVs in Bangladesh may be facilitated by the import of such materials (Adair *et al.,* 2008). The possibility of vertical transmission may is also existed because it is well known that FAdVs can be spread vertically through the embryonated egg and horizontally (Mazaheri *et al.,* 2003). Strategies should be developed to tackle the disease as different FAdV serotypes have been circulating in Bangladesh and infecting chicken. One of the control strategies may be vaccination of chicken with FAdV vaccine of specific isolated serotypes, especially to protect vertical transmission from parents to their progenies and their early life protection by maternally derived antibody (Popowich *et al*., 2018). In addition to vaccination, maintenance of farm biosecurity is essential to reduce the incidence of infection and horizontally transmission.

CHAPTER-6

CONCLUSION

In Bangladesh, the livestock sector contributes 1.90% of GDP in 2021–2022. Poultry is one of the important sub-sectors struggling with diseases. IBH results in severe depression, hydropericardium, renal inflammatory diseases, necrotizing hepatitis, increased mortality, loss of uniformity, higher feed conversion, and poor economic performance, making it an economically relevant disease. FAdV is frequently transmitted vertically through contaminated meat-type chicken breeding stock. FAdV must not be present in SPF chicken flocks, SPF eggs, or commercial vaccinations. Large numbers of chickens might unintentionally get infected in a highly consequential way if there is a possibility of SPF egg or vaccine contamination. Adenoviruses in poultry are common in several nations worldwide. When chickens without antibodies get infected, exposure to FAdV becomes considerable. Adult flocks infected with FAdV in the absence of neutralizing antibodies increase viral proliferation and shedding, leading to vertical transmission, whereas young infected chickens develop IBH. Natural exposure and/or vaccination have a major role in developing resistance to FAdV. Immune system defense and vaccination are the most effective intervention tactics. While biosecurity, disinfection, and cleaning help to maintain overall control, eliminating FAdV from commercial chicken operations is not viable from an economic or practical standpoint. FadVs (Group-1) has 12 serotypes 1 to 8a, 8b to 11, they have limited cross protection (e.g., serotype 4 vaccine cannot protect serotype 8b infection). Serotype 2, 4, 7, 10, 11, 8a, 8b are the most frequent causal agent of IBH causing second week onward high mortality. Till now, no study has demonstrated the virological, sequencing, and phylogenetic analysis of circulating agents in addition to serological evidence of FAdV infection in Bangladesh. We examined and reported the FAdVs in suspected chickens from three geographically distinct sites in Bangladesh using clinical, virological, and

serological methods in this investigation. This study provided molecular proof that three FAdV serotypes (5, 8b and 11) were spreading in Bangladeshi poultry. Additionally, serological evidence was presented. Based on the molecular detection and characterization of isolated serotypes, commercial company, vaccine producer and farmers can decide and choose the accurate IBH vaccines (multivalent or single) for immunization of the flocks. As it is a viral disease, preventive measures like immunization and the maintenance of strong biosecurity should therefore be taken into account. To combat the diseases, studies on detection of specific serotypes, selection of commercial vaccines and/or the development and manufacturing of FAdV vaccines must to be updated on a regular basis besides a comprehensive biosecurity measures.

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APPENDICES

Certificate of Ethical Approval

শিক্ষা নিয়ে গড়ব দেশ
শেখ হাসিনার বাংলাদেশ

Institute of Research and Training (IRT)
Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200

Ref. No.: HSTU/IRT/2930(1) Date: 18.06.2020

Certificate of Ethical Approval

This is to certify that, Institute of Research and Training (IRT) ensures compliance with the ethical principles of the research entitled as **"First virological evidence of fowl adenovirus (group-1) serotypes causing inclusion body hepatitis in Bangladesh".** The research work will be completed under the supervision of Professor Dr. Md. Mostafizer Rahman, Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The research methodology will be critically reviewed and checked.

Director 18.06.2020
Institute of Research and Training (IRT)
Hajee Mohammad Danesh Science and Technology University, Dinajpur

Director
Institute of Research and Training (IRT)
Hajee Mohammad Danesh Science and Technology University, Dinajpur.

Mortality Sending Form

Date:

Farm	**Unit**	**Shed**
Flock	**Age**	**Breed**

Sample Status (put √ mark) **Sample No.**

Sick Bird √ **Death Bird** Female Male

Clinical Signs

No. of Affected Birds(Visual Assumption)

LAST 7 DAYS MORTALITY			LAST 7 DAYS PRODUCTION		
Date	Female	Male	Date	Actual(%)	Standard(%)

Medication & Feed intake information

Last Antibiotics	Name	Duration by date	Dose
Last Anthelmintics	Name	Duration by date	Dose
Last 7 Days Supplementation			
Last 15 Days Vaccination	Name	Application date	Route of Adm.

Feed Clean Up Time (put √ mark)	Increase(Mentioned Time)	
Feed Consumption(g/bird)	Standard	

Serum Sending Form (Beginning of infection) Date:

FARM	UNI		SHED
FLOCK	AGE		Breed
BLEEDING DATED	SAMPLE SIZE	LAST 4WKS DATE	VACCINE NAME &
	F M		

Serum Sending Form (After recovery of infection) Date:

FARM	SPL-M	UNIT		SHED	5
FLOCK		AGE		Breed	
BLEEDING DATED		SAMPLE SIZE	LAST 4WKS DATE		VACCINE NAME &
		F M			

Nucleotide sequence of hexon gene from NIB which detected presence of stereotype-8b

GKACKGCATCGAGTCCKCSGTMCATRYYGTACGGCGGCACGGCTTACAAC
CCCCTGGCCCCTCGCGAAGCCTTCTTTAACAACTGGGTGGAGGACGAAGA
CAACAATACATCCATCACGGGGCAAATGACCAATCCGTACAAGAACGAGC
AGCAAAACACAGCTACGGCAACAGCTGGGGCAATCGCCAGCGTTTCAGGC
TCTTATCCCAACCCTAACGTGGGACTGGCCATTAGCGAAATGGGAACCCTC
ACCCCGACACTAGCAGCACAGGTTGGCCTGGCCGGACGCTTTGCCAAGGT
GTCGAGCGAGAACACGCGCCTGGCTTATGGAGCGWATGTGAAGCCTATAA
AAGACGACGGCTCTCAGTCACTTGGAACAACGCCTTACTACGTGTTAGACA
CCACCGCACAGAAATACTTGGGCGTCATGGGGGTAGAAGACTTTACGCAA
AGTCTTACCTACCCAGACAGTCTGTTAATCCCCCCTCCTTCTGAGTACAGAG
CGGTTAACAGCGGGGTGATGAAAGCCAACAGACCCAACTACATCGGWTGG
RRAAAAAAAWTWATTAAAAA

Sequences producing significant alignments with previous isolates of serotype 8b (species E)

Sequences producing significant alignments

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GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fowl adenovirus isolate FAdV/Trishal/Bangladesh/03/2022 hexon gene, partial cds	Fowl adenovirus	959	959	90%	0.0	99.81%	1062	OP688508.1
Fowl adenovirus isolate FAdV/Trishal/Bangladesh/02/2022 hexon gene, partial cds	Fowl adenovirus	959	959	90%	0.0	99.81%	1062	OP688507.1
Fowl adenovirus isolate FAdV/Trishal/Bangladesh/01/2022 hexon gene, partial cds	Fowl adenovirus	959	959	90%	0.0	99.81%	1062	OP688506.1
Fowl adenovirus 8b isolate FADV/Gazipur/Bangladesh/G09/2021 hexon gene, partial cds	Fowl adenovirus 8b	959	959	90%	0.0	99.81%	709	OP502745.1
Fowl adenovirus 8b isolate FAdV/Panchgarh/Bangladesh/A03/2022 hexon gene, partial cds	Fowl adenovirus 8b	959	959	90%	0.0	99.81%	581	OP454908.1
Fowl adenovirus 8b isolate FADV/Gazipur/Bangladesh/08714/2022 hexon gene, partial cds	Fowl adenovirus 8b	959	959	90%	0.0	99.81%	709	OP004931.1
Fowl aviadenovirus E isolate TH/KKU05/2021IBH hexon protein gene, partial cds	Fowl aviadenovirus E	953	953	90%	0.0	99.62%	537	OK078886.1
Fowl aviadenovirus E isolate TH/KKU04/2021IBH hexon protein gene, partial cds	Fowl aviadenovirus E	953	953	90%	0.0	99.62%	537	OK078885.1
Fowl aviadenovirus E isolate TH/KKU03/2021IBH hexon protein gene, partial cds	Fowl aviadenovirus E	953	953	90%	0.0	99.62%	537	OK078884.1
Fowl aviadenovirus E isolate TH/KKU02/2021IBH hexon protein gene, partial cds	Fowl aviadenovirus E	953	953	90%	0.0	99.62%	537	OK078883.1
Fowl aviadenovirus E isolate TH/KKU01/2021IBH hexon protein gene, partial cds	Fowl aviadenovirus E	953	953	90%	0.0	99.62%	532	MZ779124.1
Fowl aviadenovirus E isolate THV/KKU/2021 hexon protein gene, partial cds	Fowl aviadenovirus E	950	950	90%	0.0	99.61%	531	OP434336.1
Fowl aviadenovirus E isolate THP/KKU/2021 hexon protein gene, partial cds	Fowl aviadenovirus E	950	950	90%	0.0	99.61%	531	OP434335.1
Fowl aviadenovirus E isolate THKL17/KKU/2021 hexon protein gene, partial cds	Fowl aviadenovirus E	950	950	90%	0.0	99.61%	531	OP434334.1
Fowl aviadenovirus E isolate THKL3/KKU/2021 hexon protein gene, partial cds	Fowl aviadenovirus E	950	950	90%	0.0	99.61%	531	OP434333.1
Fowl aviadenovirus E isolate THNR/KKU/2021 hexon protein gene, partial cds	Fowl aviadenovirus E	950	950	90%	0.0	99.61%	531	OP434332.1
Fowl adenovirus 8b strain FAV8b/Hubei/duck/H2138/2019 hexon protein gene, partial cds	Fowl adenovirus 8b	931	931	90%	0.0	98.85%	749	ON502589.1
Fowl aviadenovirus E isolate BIO5 hexon gene, partial cds	Fowl aviadenovirus E	926	926	90%	0.0	98.66%	813	KX755572.1
Fowl adenovirus 8b strain FAV8b/Jiangsu/chicken/J2196/2019 hexon protein gene, partial cds	Fowl adenovirus 8b	926	926	90%	0.0	98.66%	749	ON502593.1
Fowl aviadenovirus E isolate vsn033pat17 hexon gene, partial cds	Fowl aviadenovirus E	920	920	90%	0.0	98.46%	720	MK692960.1
Fowl adenovirus DDO-2007 isolate 04-53357-122 hexon protein gene, partial cds	Fowl adenovirus DDO-2007	920	920	90%	0.0	98.46%	1247	EF685489.1
Fowl adenovirus DDO-2007 isolate 06-41265-31 hexon protein gene, partial cds	Fowl adenovirus DDO-2007	915	915	90%	0.0	98.27%	813	EF685622.1
Fowl adenovirus DDO-2007 isolate 04-18871_03-10572 hexon protein gene, partial cds	Fowl adenovirus DDO-2007	915	915	90%	0.0	98.27%	575	EF685541.1
Fowl adenovirus DDO-2007 isolate 04-18871_04-10058 hexon protein gene, partial cds	Fowl adenovirus DDO-2007	915	915	90%	0.0	98.27%	575	EF685538.1
Fowl adenovirus DDO-2007 isolate 04-52486 hexon protein gene, partial cds	Fowl adenovirus DDO-2007	915	915	90%	0.0	98.27%	855	EF685515.1

Nucleotide sequence of hexon gene from NIB which detected presence of stereotype-11

GCTTGCAGAGGACTTCTTTTAACCAGTATGGAGGACCGCATACAATCCCCT
CGCGCCCCGCGAAGCCTYTTTTCAACAATTGGGTTGACACAGAGGCGAGC
AAGACCGTCATCACGGGTCAGATGACAACTCCCTACGAAAACGTCCAGGG
CGCTAAAGACAAGACTGCCGCGATCGTCGCCGCTCTTTCAGGGGTTTATCC
CGATCCCAATATCGGTACCGCCATCAGCGAGATGGGCGCCTTAGACGCGAC
GTCGGCAGCCCAAGTCGGATTGGCTGCCCGATTCGCGAAAGTGTCGAGCG
ATAACACGCGTCTAGCCTACGGAGCCTACGTTAAACCGCTCAAGAACGACG
GTTCTCAATCGATTAACCCCACTCCTTACTGGGTCATGGACAGCAACGCCA
CAAACTATCTCGGAGTCATGGGAGTCGAAGACTTTAGCGCCTCGCTAACCT
ATCCCGATACGCTCCTCATTCCCCCGCCGACCGAATACTCAGAAGTGAATAC
CGGCGTCATGAAGGCRAACAGGCCGAATTACATCGKCGGRAAAAAAWWW
WWAAAAAAAAG

Sequences producing significant alignments with previous isolates of serotype 11(species D)

Sequences producing significant alignments Download Select columns Show 100

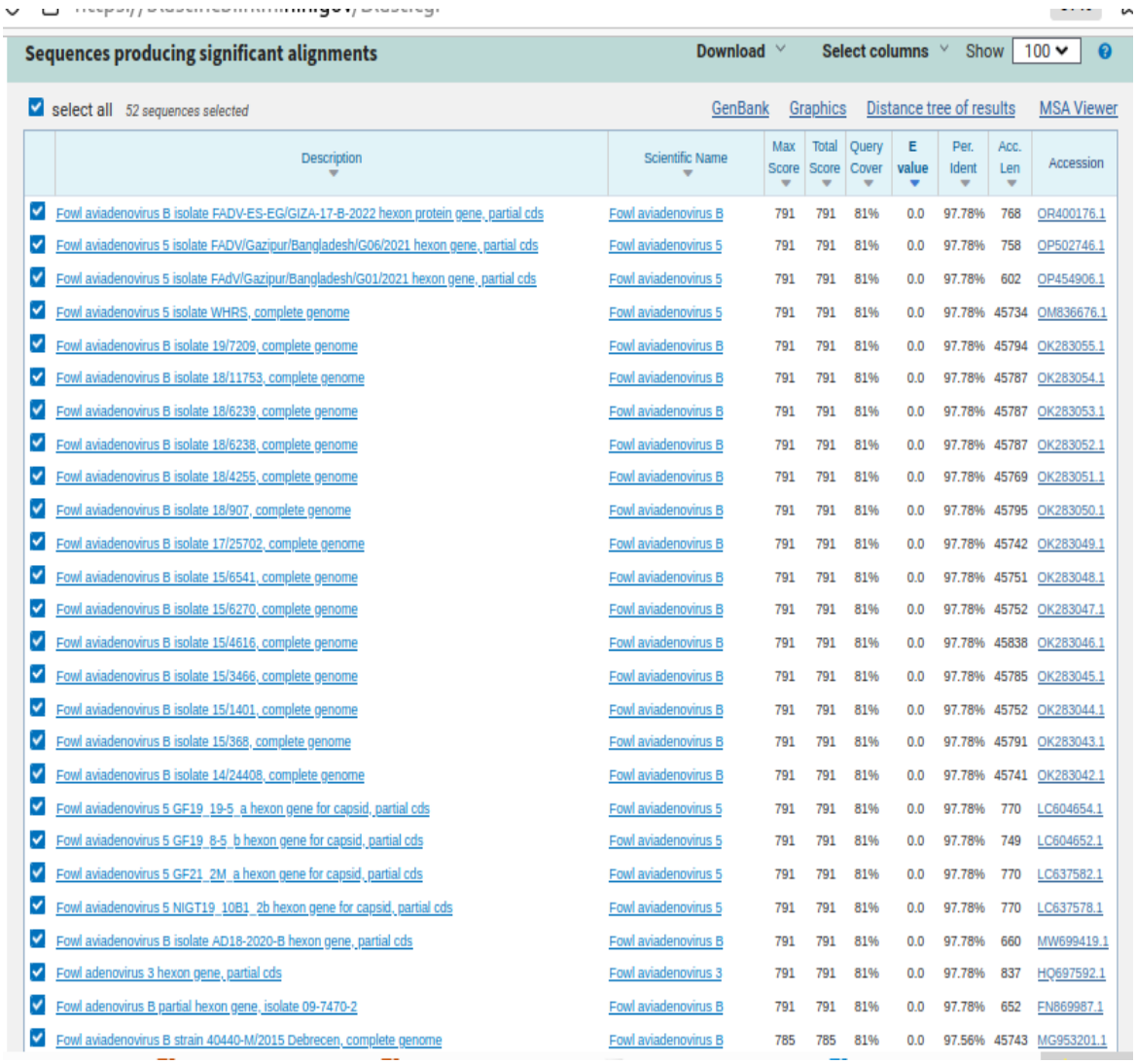
select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fowl aviadenovirus 11 strain ADL15 0666 hexon protein gene, partial cds	Fowl aviadenovirus 11	963	963	94%	0.0	98.89%	723	MN737046.1
Fowl aviadenovirus D isolate FAdV-SAC104 hexon gene, complete cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	2856	MK995483.1
Fowl aviadenovirus D isolate FAdV-SAC101 hexon gene, complete cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	2853	MK995481.1
Fowl aviadenovirus D strain 12-11324, complete genome	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	43405	MK572872.1
Fowl aviadenovirus D isolate TR/BVKE/R/AC-2 hexon gene, partial cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	849	MK937072.1
Fowl adenovirus JP/Miyagi/2010IBH L3 gene for hexon protein, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	748	LC505995.1
Fowl adenovirus JP/Iwate/2010IBH L3 gene for hexon protein, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	748	LC505994.1
Fowl adenovirus isolate M12 hexon gene, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	759	MK642677.1
Fowl adenovirus strain MOR300315 hexon protein gene, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	694	MK468898.1
Fowl aviadenovirus A isolate TTSR05_2015 hexon gene, partial cds	Fowl aviadenovirus A	963	963	94%	0.0	98.89%	854	MG676337.1
Fowl aviadenovirus A isolate TTAR01_2015 hexon gene, partial cds	Fowl aviadenovirus A	963	963	94%	0.0	98.89%	855	MG676334.1
Fowl aviadenovirus A isolate TTAA011_2015 hexon gene, partial cds	Fowl aviadenovirus A	963	963	94%	0.0	98.89%	865	MG676333.1
Fowl adenovirus isolate FAV-YT-160809-B hexon gene, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	847	MF573933.1
Fowl adenovirus isolate FAV-QDJM-151112 hexon gene, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	850	MF573920.1
Fowl adenovirus isolate FAV-LNDL-160711-B hexon gene, partial cds	Fowl adenovirus	963	963	94%	0.0	98.89%	846	MF573911.1
Fowl aviadenovirus D isolate SD15-24 hexon protein gene, complete cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	2853	KY426992.1
Fowl aviadenovirus D isolate LN15-2 hexon protein gene, complete cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	2853	KY426985.1
Fowl aviadenovirus D isolate LN15-1 hexon protein gene, complete cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	2853	KY426983.1
Fowl aviadenovirus D isolate JL/1407, complete genome	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	44054	KY012057.1
Fowl aviadenovirus D isolate S1763-1_D2, complete genome	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	43913	ON260920.1
Fowl aviadenovirus D isolate DDLSL21 genomic sequence	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	43339	OQ749511.1
Fowl aviadenovirus D NS-5_2019 L gene for hexon protein, partial cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	727	LC760354.1
Fowl aviadenovirus D Ns-2_2017 L gene for hexon protein, partial cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	694	LC760348.1
Fowl aviadenovirus D NS-1_2017 L gene for hexon protein, partial cds	Fowl aviadenovirus D	963	963	94%	0.0	98.89%	736	LC760347.1
Fowl aviadenovirus 11 isolate FAdV/Panchgarh/Bangladesh/T02/2021 hexon gene, partial cds	Fowl aviadenovirus 11	963	963	94%	0.0	98.89%	579	OP454907.1

Nucleotide sequence of hexon gene from NIB which detected presence of stereotype-5 by NCBI blasting

GRCATMGTCGACWTCGGGAATATSYATCGRCTACGGGWMYATCATSCWCW
MGGGAGGAGAGCCTCYGCKAGAAGTGCGACGAGCAAYAGGAGAGGTTCA
CCGGCAAATGACCCATCCTTATGCTAACGAGACCAACACCAATCCTACGAA
GACGGCCGCCGCATCRSCAGSATGTCCGGCACCGTTCCCAATCCTAACCTG
GGACCGTGCATTAGCGAGATGTCCGCGCTGGMTACTACCAGCGCCGATAGT
GTAGGACTTATGGCCCGTTTCGCCAAGATAGGAGCCACGAACAACAAATTA
GCCTACGGAGCGTACGTGAAACCGGTGAACAACGACGGTTCGCAGTCGCT
GACCCAAACGGCTTATTGGGTAACGGATAACTCGGATGCTAATTATTTGGGA
GCTCTGTCGGTAGAAGACTTTACCACGAGCCTGACGTATCCGGACAGTCTT
CTCATTCCGGCGCCTACCGMATACTCCAACGTCAATAACGGAACGATGAAG
GCCAACCGTCCCAACTACATCGGMTTYMGGGACAATTTSAA

Sequences producing significant alignments with previous isolates of serotype 5(species B)



Sequences producing significant alignments Download Select columns Show 100

select all 52 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fowl aviadenovirus B isolate FADV-ES-EG/GIZA-17-B-2022 hexon protein gene, partial cds	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	768	OR400176.1
Fowl aviadenovirus 5 isolate FADV/Gazipur/Bangladesh/G06/2021 hexon gene, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	758	OP502746.1
Fowl aviadenovirus 5 isolate FAdV/Gazipur/Bangladesh/G01/2021 hexon gene, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	602	OP454906.1
Fowl aviadenovirus 5 isolate WHRS, complete genome	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	45734	OM836676.1
Fowl aviadenovirus B isolate 19/7209, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45794	OK283055.1
Fowl aviadenovirus B isolate 18/11753, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45787	OK283054.1
Fowl aviadenovirus B isolate 18/6239, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45787	OK283053.1
Fowl aviadenovirus B isolate 18/6238, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45787	OK283052.1
Fowl aviadenovirus B isolate 18/4255, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45769	OK283051.1
Fowl aviadenovirus B isolate 18/907, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45795	OK283050.1
Fowl aviadenovirus B isolate 17/25702, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45742	OK283049.1
Fowl aviadenovirus B isolate 15/6541, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45751	OK283048.1
Fowl aviadenovirus B isolate 15/6270, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45752	OK283047.1
Fowl aviadenovirus B isolate 15/4616, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45838	OK283046.1
Fowl aviadenovirus B isolate 15/3466, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45785	OK283045.1
Fowl aviadenovirus B isolate 15/1401, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45752	OK283044.1
Fowl aviadenovirus B isolate 15/368, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45791	OK283043.1
Fowl aviadenovirus B isolate 14/24408, complete genome	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	45741	OK283042.1
Fowl aviadenovirus 5 GF19_19-5_a hexon gene for capsid, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	770	LC604654.1
Fowl aviadenovirus 5 GF19_8-5_b hexon gene for capsid, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	749	LC604652.1
Fowl aviadenovirus 5 GF21_2M_a hexon gene for capsid, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	770	LC637582.1
Fowl aviadenovirus 5 NIGT19_10B1_2b hexon gene for capsid, partial cds	Fowl aviadenovirus 5	791	791	81%	0.0	97.78%	770	LC637578.1
Fowl aviadenovirus B isolate AD18-2020-B hexon gene, partial cds	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	660	MW699419.1
Fowl adenovirus 3 hexon gene, partial cds	Fowl aviadenovirus 3	791	791	81%	0.0	97.78%	837	HQ697592.1
Fowl adenovirus B partial hexon gene, isolate 09-7470-2	Fowl aviadenovirus B	791	791	81%	0.0	97.78%	652	FN869987.1
Fowl aviadenovirus B strain 40440-M/2015 Debrecen, complete genome	Fowl aviadenovirus B	785	785	81%	0.0	97.56%	45743	MG953201.1

Certificate of Appreciation from international poultry seminar (WPSA-BB) due to an oral presentation on Research Work

education organization research
World's Poultry Science Association, Bangladesh Branch

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