USMANU DANFODIYO UNIVERSITY, SOKOTO

(POSTGRADUATE SCHOOL)

SERO-PREVALENCE AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS IN CHICKENS IN SOKOTO STATE NIGERIA

A Thesis

Submitted to the

Postgraduate School,

USMANU DANFODIYO UNIVERSITY, SOKOTO, NIGERIA

In partial fulfillment for the Degree of

DOCTOR OF PHILOSOPHY (VETERINARY MEDICINE)

By

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JUNE, 2018

DEDICATION

This work is dedicated to my late father Alhaji Umaru Mungadi and my passionate mother Hajiya Fatima Umaru Mungadi who struggled in life to see that I became what I am today with the help of Almighty Allah.

CERTIFICATION

This Thesis by MUNGADI, Hauwa'u Umar (14310803001) has met the requirements for the award of the degree of Doctor of Philosophy (Veterinary Medicine), of the Usmanu Danfodiyo University, Sokoto, and is approved for its contribution to knowledge.

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ACKNOWLEDGEMENTS

My sincere gratitude is to the Almighty Allah who in his infinite mercy gave me the strength and knowledge to carry out this research. The support and cooperation of my supervisors; Dr. U.M. Mera, Prof. A.I. Daneji and Dr. U. Musa throughout this work is highly appreciated.

My appreciation goes to Dr. Faruku Bande Yabo of Ministry of Animal Health and Fisheries, State Veterinary Clinic, Sokoto and Dr. B.R. Alkali of Department of Veterinary Microbiology for their assistance and contributions towards the success of this work. I also appreciate Mal. A.B. Shu'aibu Department of Veterinary Microbiology, Dr. S. Sama'ila Department of Veterinary Pharmacology, Dr. Y. Yakubu Department of Veterinary Public Health and Preventive Medicine and Dr. Emeka Ndodo Coordinator Center for Advance Medical Research and Training (CAMRET) Usmanu Danfodiyo University Teaching Hospital (UDUTH) Sokoto, for their immense contributions and support during my laboratory work. I also thank go to Dr. S. Gatawa Ministry of Animal Health and Fisheries, State Veterinary Clinic, Sokoto, Dr.s A. Ahmed H.O.D Theriogenology and Animal production, Garba Sirajo and U.S. Ahmad Veterinary Teaching Hospital UDUS for the assistance, support and encouragement they rendered to me in the course of this work.

I would also like to acknowledge my aunty Hajiya Amina Abubakar Zaki, my cousin Yusuf Abubakar Zaki and the entire family of Late Alhaji Umar Mungadi whose constant prayer, endless love and support with the will of Almighty Allah made this work a success.

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LIST OFACRONYMS

ABC ---------- Avidin-biotin complex

AGID ---------- Agar gel immunodiffusion

AIV ---------- Avian Influenza Virus

ALV ---------- Avian Leucosis Virus

APV ---------- Avian Pneumovirus

ArK ---------- Arkansas

ATI ---------- Alveolar type I

ATII ---------- Alveolar type II

cDNA ---------- Complimentary DNA

CIS ---------- Cross Immunization Study

DDBJ ---------- DNA Data Bank of Japan

E ---------- Envelop

ECE ---------- Embryonated chicken egg

EDS ---------- Egg Drop Syndrome

EDTA ---------- Ethylenediaminetetraacetic acid

ELISA ---------- Enzyme Linked Immunosorbent Assay

EMBL ---------- European Molecular Biology Laboratory

HI ---------- Hemaglutination Inhibition

HVR ---------- Hyper variable region

IB ---------- Infectious Bronchitis

IBD ---------- Infectious Bursa Disease

IBV ---------- Infectious Bronchitis Virus

IC ---------- Infectious Coryza

IDT ---------- Integrated DNA Technologies

IFAT ---------- Indirect Fluorescent Antibody Test

IFA ---------- Indirect Fluorescent Antibody Assay

ILTV ---------- Infectious Laryngo tracheitis Virus

M ---------- Membrane

MAHFD ---------- Ministry of Animal Health and Fisheries Development

Mass ---------- Massachusetts

MD ---------- Marex Disease

MEGA ---------- Molecular Evolutionary Genetics Analysis

MG ---------- Mycoplasma Gallisepticum

MUSCLE ---------- Multiple Sequence Comparison with Log Expectation

N ---------- Nucleocapsid

NCBI ---------- National Center for Biotechnology Information

ND ---------- Newcastle Disease

NGS ---------- Next generation sequencing

ORF ---------- Open reading frame

RBD ---------- Receptor binding domain

RE ---------- Restriction endonuclease

RFLP ---------- Restriction Fragment Length Polymorphism

RNA ---------- Ribo Nucleic Acid

RT-LAMP ---------- Reverse transcriptiom loop-mediated isothermal amplification

RT-PCR ---------- Reverse Transcriptase Polymerase Chain Reaction

S ---------- Spike

SARS ---------- Severe Acute Respiratory Syndrome

SPF ---------- Specific pathogen free

TBE ---------- Tris-borate EDTA

TEM ---------- Transmission electron microscopy

TOC ---------- Tracheal organ culture

UTR ---------- Untranslated Region

UV ---------- Ultra violet

VNT ---------- Virus neutralization test

ABSTRACT

In this study, infectious bronchitis virus (IBV) was investigated. Blood samples were collected from 400 chickens for serology using enzyme linked immunosorbent assay (ELISA). Also tracheal and cloacal swabs were collected from 150 chickens for reverse transcription polymerase chain reaction (RT-PCR), gene sequencing and Phylogenetic analysis. The overall sero-prevalence and RT-PCR positives obtained were 86.50% and 13.09% respectively. Sokoto zone had the highest percentage of RT-PCR positives (63.64%). The seroprevalence of IBV in indigenous and exotic chickens were 88.72% and 84.39% respectively showing statistically insignificant association using chi square, P= 0.205 (>0.05%). RT-PCR result showed higher positives in indigenous chickens (63.64%) than in exotic (36.36%). The seroprevalence in young and adult chickens were 89.50% and 82.87% respectively with significant association; P= 0.038 (<0.05), binary logistic regression showed no significant relationship with P= 0.09 (>0.05). RT-PCR result showed higher positives in young (72.73%) than in adults (27.27%). In male and female chickens, the seroprevalence were 86.88% and 86.17% respectively with no significant statistical association, P= 0.836 (>0.05%). RT-PCR result showed higher positives in males (54.55%) than in females (45.45%). Sequencing and phylogenetic analysis showed four of the strains isolated were close to NGA/A11637/2006 strain while one of the sequences showed closeness to patridge/GD/S14/2003 strain. This study has provided an update on seroprevalence of IBV and some circulating IBV strains in the study area. In conclusion there is high seroprevalence of IBV in chickens in Sokoto State. RT-PCR result showed low prevalence of the virus. The isolated strains are variants of patridge/GD/S14/2003 and NGA/A11637/2006 strains. This is the first study where patridge/GD/S14/2003 like strain is reported in Nigeria. Antigen detection ELISA kit

is recommended for serological survey of IBV in future researches. There is need to know the pathogenicity of the circulating IBV strains and test the efficacy of available vaccines.

CHAPTER ONE

INTRODUCTION

1.1 Study Background

Infectious bronchitis (IB) is an acute, highly contagious viral disease of chickens caused by infectious bronchitis virus (IBV), which is classified as an enveloped, single-stranded RNA virus of the genus *gamma Coronavirus*, family *Coronaviridae* (Gary *et al.,* 2009).

The *Coronaviridae* family has been grouped together with the *Arteriviridae* into the order Nidovirales, the two virus families have many similarities (Hiscox *et al*., 2001). More than 50 serotypes within the IBV are recognized worldwide (Lee and Jackwood, 2000; McKinley *et al.,* 2008, Bande *et al.,* 2017). The disease is of economic importance and distributed worldwide (Gary *et al.,* 2009). The incidence of the disease is not constant throughout the year, being reported more often during the cooler months (Gary *et al.,* 2009). Reports suggest a shift in tissue tropism, and an extended host range into bird species such as Chinese peafowl (*Pavo cristatus*), guinea fowl (*Numida meleagris*), partridge (*Alectoris* species) and teal (*Anas* species) reared close to domestic fowl (*Gallus domesticus*) (Yu *et al.,* 2001; Zhou *et al.,* 2004; Liu and Kong, 2004; Liu *et al.,* 2006). Until recently, the chicken was considered the only natural host of IBV and in which the virus cause disease. Pheasants are the other avian species that is now considered as a second natural host for IBV (Ignjatovic and Sapats, 2000).

The virus is spread via the respiratory route in droplets expelled during coughing or sneezing by infected chickens, likewise infection may occur through

ingestion of contaminated feed or water (Ignjatovic and Sapats, 2000). IBV causes significant economic losses, mostly because of reduced productivity rather than bird mortality (Cavanagh and Naqi, 2003). Morbidity rates may vary from 50-100% and mortality rates range from 0-25% depending on secondary infections especially with bacterial pathogens (Anon, 2005). According to Anon (2012) when infectious bronchitis occurs in a laying flock, production usually drops to near zero within few days to four weeks. This trend persists before the flock returns to normal production though some flocks never regain an economical rate of lay.

The incubation period of IB is usually 8-48 hours (Gary *et al.,* 2009). The disease is characterized by respiratory signs such as gasping, coughing, sneezing, tracheal rales and nasal discharge (Whiteman and Bicford, 1996; Gary *et al.,* 2009). Other signs include depression, swollen face and frothy exudate in the eyes. In layers, there are respiratory distress, decrease in egg production and loss of internal egg quality and egg shell quality (Gary *et al.,* 2009). Lesions associated with IB include consolidation of the lungs, inflammation of the trachea, inflammation of the ureters and presence of urates in the ureters (Worthington *et al.,* 2008).

The differential diagnosis of the disease represents a challenge with respect to other respiratory diseases caused by *Mycoplasma gallisepticum* (chronic respiratory disease), infectious laryngotracheitis virus, *Haemophilus paragallinarum* (infectious coryza) and Newcastle disease virus **(**Ducatez *et al.*, 2009).

Diagnosis of IB is achieved by serological techniques including Enzyme Linked Immunosorbent Assay (ELISA), Virus Neutralization Test (VNT), and Hemagglutination Inhibition (HI). Molecular techniques include Restriction Fragment Length Polymorphism (RFLP) and Reverse Transcription Polymerase Chain Reaction

(RT-PCR) (Gary *et al.*, 2009; Bande *et al*., 2016). The ELISA assay is a convenient method for monitoring of both the immune status and virus infections in chicken flocks. Several commercial ELISA kits for IBV specific antibodies detection are already available which use inactivated virions as coating antigen (Zhang *et al.*, 2005). RT-PCR on reverse transcribed RNA is a potent technique for the detection of IBV viral RNA and comparison with classical detection methods; it offers both sensitive and fast detection (Zwaagstra *et al*., 1992). Virus isolation has been recognized as gold standard and samples frequently used include swab materials from the trachea, lungs and kidneys of infected chickens (Sylvester *et al*., 2003a; Dhama *et al*., 2011a, b; Fan *et al*., 2012). Samples for IBV isolation are obtained as soon as clinical signs of the disease are evident. Tracheal swabs are preferred and are placed directly into cold media with antibiotics to suppress bacterial growth and preserve the viability of the virus (Swayne *et al*. 1998).

Infectious Bronchitis has been reported as a disease of chickens of all ages; however, severity of the disease varies with strains of the virus (Gary *et al.*, 2009). The emergence of a *Coronavirus* variant causing severe acute respiratory syndrome (SARS) in humans has renewed interest in the virus family *Coronaviridae* (Cavanagh, 2000). IBV and other avian coronaviruses of turkeys and pheasants are classified as group 3 coronaviruses, with mammalian coronaviruses comprising groups 1, 2 and 4. Group 4 is the more recently identified severe acute respiratory syndrome (SARS) Coronavirus (Cavanagh, 2000; Enjuanes *et al.*, 2000; Lai and Holmes, 2001). Turkeys can be infected by group 2 as well as group 3 coronaviruses (Lai and Holmes, 2001). So far, group 3 viruses have not been found in humans, but phylogenetic analysis of SARS-coronavirus has shown that its genome contains sequences that seem to be of group 3 in origin (Stavrinides and Guttman, 2004).

Isolation and identification of the virus is achieved by inoculation in chicken embryos. There is no specific treatment for this disease (Anon., 2012). Sodium salicylate at 1g/litre of drinking water has been used in the acute phase of the disease as analgesic, anti-inflammatory and antipyretic, antibiotics have been used to control secondary bacterial infections such as colibacillosis (Paul, 2004). Prevention of IB is best achieved through effective biosecurity measures (Worthington *et al,* 2008; Gary *et al.,* 2009). Vaccines against IB have arguably been the most successful and the most widely used vaccines for diseases caused by Coronaviruses (Cavanagh, 2003).

The IB virus constitutes one of the most important viruses of poultry because of its numerous serotypes and lack of cross protection, (Worthington *et al.,* 2008). Some strains of IBV are quite effective in inducing cross protection against other serotypes and are referred to as protectotypes eg. M41 a prototype of Massachusetts serotype group (Worthington *et al.,* 2008).

Four virus-specific proteins have been identified; the spike (S) glycoprotein, the membrane or matrix (M) glycoprotein, the envelope (E) protein and the nucleocapsid (N) protein (Shi *et al.,* 1999). The nucleocapsid (N) protein of IBV is closely associated with the genomic RNA, and has highly conserved amino acid and nucleotide sequences, i.e. very little variation in the N-gene sequence is seen between various strains of IBV. The spike glycoprotein (S) is anchored in the viral envelope and is post translationally cleaved into two proteins designated S1 and S2. In contrast to the N protein, the S1 segment protein is very diverse in terms of both nucleotide sequence and deduced amino acid sequences (Ignjatovic and Galli, 1994; Cavanagh, 1995). It is believed that important neutralizing antibody-inducing epitopes are situated in the S1 protein, thus it is essential for development of protective immunity.

Thus, a common identification of IBV strains can obviously apply to the nucleoprotein gene sequence, while genotypic characterization largely depends on the hyper variable region of S1 glyco-protein (Ignjatovic and Galli, 1994).

It is important to isolate and type the IBV virus strains prevailing in a geographical area regularly and vaccinate birds accordingly with vaccine strains that offer maximum cross protection against the circulating/prevailing field virus. Combination of different strains may provide broad protection in comparison with single strain based vaccine. Similarly, vaccines can be developed from recently isolated IBV field strain(s) from a particular region (Shi *et al*., 2006). Live attenuated vaccines are most often used in the vaccination program; however it is plagued with limitations including poor thermostability, reversion to virulence, and recombination between vaccine and field viruses (Tarpey *et al.*, 2006; McKinley *et al.*, 2008; Lee *et al.*, 2010, 2012; Bande *et al.*, 2015). These factors may have contributed to the increased emergence of genetically diverse IBV strains that undermines efforts in the control of the disease (Bande *et al.*, 2015). The new generation vaccines (recombinant and vector-based) developed against locally prevailing IBV strains may be more helpful especially to avoid the reversion to virulence which is associated with live vaccines (Kuldeep *et al.*, 2014).

In North America, common serotypes used in vaccination programs include the Massachusetts, Connecticut, and Arkansas serotypes (Gary *et al.*, 2009). H120 vaccine has been successfully used worldwide as a primary vaccine for broilers and for initial vaccination of breeders and layers. Moreover, the H strains have the ability to induce at least some cross-protection against heterologous serotype viruses, providing a broader range of immunity. Inactivated oil-emulsion IBV vaccines have

also been developed that give long-lasting immunity to laying hens to prevent drops in egg production (Cavanagh and Naqi, 2003). However, in spite of good vaccines and rigorous vaccination programs IB remains a problem (Smati *et al.*, 2002; Jackwood *et al.*, 2005). This in part, is associated with the virus tendency to mutate and recombine thus leading to new viral genotypes (Wang *et al,* 1993). Outbreaks of IB are often due to infections with the strains serologically different from the strains used as vaccines. Outbreaks mainly occur during winter (Lopez and McFarlane, 2006).

Commonly known IBV serotypes include the Massachusetts (Mass) type which was first isolated in USA in the 1940s (Cavanagh and Davis, 1993). The D274-type was the most reported in several Western European countries in the early and mid-1980s (Cook, 1984; Cavanagh *et al*., 1992). Whereas 4/91-type or CR88 was first isolated in 1985 in France; later its type was identified in the United Kingdom (UK) (Cook *et al*., 1996).

Handberg *et al*. (1999) carried out a research in Denmark to detect and differentiate strains of IBV in tracheal tissue by reverse transcription polymerase chain reaction (RT-PCR). All reference IBV strains employed (M41, H52, H120, Beaudette, Connecticut, D1466, D274, D3896, Arkansas 99, 793B, JMK) in allantoic harvests were detected.

Di Fabio *et al*. (2000) characterized infectious bronchitis viruses in Brazil after isolation from poultry flocks that were experiencing IB like conditions. One of the isolates was the Massachusetts serotype. The remaining were examined by means of cross-neutralization tests in tracheal organ cultures and were shown to belong to at

least four antigenic groups, all different from ones described previously in other countries.

In Jordan, survey on seroprevalence and detection of infectious bronchitis virus was carried out by Dergham *et al*. (2009) in commercial chicken flocks. Respiratory disease free chickens were screened for the presence of Massachusetts-41 (M41), D274, and 4/91 strain antigens of IBV by using hemagglutination inhibition (HI) test. Reverse transcription polymerase chain reaction (RT-PCR) technique was used also to test chickens showing signs of respiratory disease; the virus was detected in 58.8% of the chicken flocks.

In early 2011, respiratory disease outbreaks were investigated in Iraq, Jordan, and Saudi Arabia. Five IBV isolates designated JOA2, JOA4, Saudi-1, Saudi-2, and Iraqi IBV were detected by nested PCR assay. Strain identification was characterised by sequencing and phylogenetic analysis of the amplified hypervariable region of the spike 1 (S1) gene. These five IBV isolates were found to be of the IBV strain CK/CH/LDL/97I (Ababneh *et al*., 2012).

Boroomand *et al.* (2012) reported in their study that infectious bronchitis virus isolate IRFIBV32 which was recently isolated in Iran, when inoculated intranasal in chicks elicited mild tracheal rales, coughing, and gasping; IRFIBV32 also induced mild lesions in trachea and kidneys.

Similarly, Mohammad *et al.* (2013) conducted a study in Zabol, south east of Iran for the detection of infectious bronchitis virus serotypes by reverse transcription polymerase chain reaction (RT-PCR) in broiler chickens. The results of the study showed that 36.36% of the sampled flocks were positive to IBV by RT-PCR.

Moreover, the Massachusetts was the identified serotype of the virus. The results provided the first molecular evidence for the presence of infectious bronchitis virus and Massachusetts serotype in Zabol.

On the African continent, IB was observed after several studies in different in countries (El Houadfi and Jones, 1985; Kelly *et al*., 1994; Thekisoe *et al*., 2003; Owoade *et al*., 2004, 2006; Mushi *et al*., 2006; Ducatez *et al*., 2009; Selim *et al.*, 2013; Mungadi *et al*., 2015). In Nigeria, IBV was established after serological survey by Komolafe *et al*. (1990), Owoade *et al*. (2004, 2006) and Mungadi *et al*. (2015) and through molecular techniques by Ducatez *et al*. (2009). Selim *et al.* (2013) carried out phylogenetic analysis based on the obtained sequences of 13 IBV isolates from Egypt; and compared them with other IBV strains from the Middle East and worldwide. Results revealed that 11 out of the 13 isolates had close relationship with the Israeli variants (IS/885 and IS/1494/06), nucleotide homology was found to reach up to 89.9% and 82.3%, respectively. Only two isolates had close relationship with CR88 or 4/91 viruses with identities of 95% and 96%, respectively. This study indicates existence of two variant groups of IBV circulating in Egypt during 2012. Group I was similar but distinguishable from Israeli variant IS/885 and group II was related to 4/91 and CR88 vaccine strains. There was no geographical link between the two groups as they were distributed all over the country.

Khataby *et al.* (2016), reported that IBV variants reported in Africa, display a low genetic relationship between them and with the majority of the reference strains emerging in neighboring countries, except the case of variants from Libya and Egypt that show a high relatedness. But Fellahi *et al*. (2015b) reported ITA 02 strain in Morocco in the first study in which Italy 02 types have been detected in Morocco and

in Africa. They reported that geographical proximity and close trans border relation between Morocco and Europe may explain in part the dominance of this genotype in the north and central regions of Morocco.

In another study, Ducatez *et al.* (2009) analyzed the presence of IBV strains in poultry in some West African countries (Niger and Nigeria) using virus cross-neutralization test and RT-PCR. The results showed not only that common IBV strains such as 793/B-, Massachusetts-, D274- and B1648-like strains circulate in the region as well as a new strain designated as "IBADAN" NGA/A116E7/2006 which is a QX-like IBV variant with unusual genetic and serological characteristics. It was suggested that it is a distinct variant unique to Nigeria.

Since the reports of Ducatez *et al*. (2009) on the strains of IBV in some states in Nigeria (Sokoto inclusive), there was dearth of information on the prevailing strains of the virus in this environment which necessitates carrying out research to know the current situation of the virus.

1.2 Statement of the Problem

The continuous emergence of new IBV serotypes and lack of cross protection among the different serotypes cause challenges as well as serious threat to effective control of the disease especially in developing countries. (Worthington *et al*, 2008, Bande *et al.,* 2015). This leads to serious economic losses in the poultry industry and generally affecting protein supply worldwide (De Wit *et al.,* 1998).

The inherent properties of the virus to change as a result of mutation, gene rearrangement and recombination further add to the challenge of controlling the disease (Wang *et al.,* 1993).

The simultaneous presence of vaccine and variant field strains of IBV in poultry population complicates the diagnostic procedures (Cavanagh and Naqi, 2003). The vaccination programs are constantly being challenged, thus even though the disease was first reported more than 70 years ago, its control is still a challenge (Cook *et al.*, 2012). This necessitates knowing the circulating IBV strains to guide the choice of vaccines. There is no update on the epidemiology of the disease in Nigeria specifically in Sokoto State.

1.3 Justification of the Research

Ducatez *et al*., (2009) reported a new genotype in Nigeria, also Mungadi *et al*. (2015) reported high seroprevalence of IBV in chickens in Sokoto state (89%). For routine surveillance of the disease, it is important to know its current status.

The circulating IBV serotypes in this environment must first be determined before deciding the use of a vaccine. Knowing the circulating IBV strains in an environment enable the formulation of an effective vaccine that will protect against the prevalent strains in that environment, as well as enable prediction of viral mutation and emergence of wild strains (Kuo *et al*., 2010).

The result of this study will add to the limited information on circulating IBV strains in this environment.

1.4 Hypotheses

1.4.1 Null Hypothesis: IBV strains circulating among local and commercial chickens in Sokoto State have already been reported elsewhere in the world.

1.4.2 Alternative Hypothesis: There are new IBV strains circulating in Sokoto state that have not been previously reported.

1.5 Aim and Objectives

1.5.1 Aim: The main aim of this study was to determine the seroprevalence of IBV and to characterize the strains of the virus circulating in Sokoto State using molecular techniques.

1.5.2 Objectives

The specific objectives of the study were to:

- Determine the presence of antibodies to IBV in Chickens in Sokoto State.
- Detect and apply molecular technique to characterize IBV isolated from chickens in Sokoto State.
- Determine the evolutionary relationship and genetic diversity of IBV strains in Sokoto State.
- Evaluate age, sex and type specific prevalence of IBV in chickens in Sokoto state.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Infectious bronchitis is an infectious and contagious disease that mainly affects the renal, reproductive and enteric systems of breeders, layers and broilers, caused by a great diversity of avian infectious bronchitis virus (IBV) which has worldwide distribution (Worthington *et al.,* 2008). Whiteman and Bicford (1996) defined infectious bronchitis as an acute, highly contagious, viral disease of chickens characterized by respiratory signs (gasping, sneezing, coughing and nasal discharge), severe renal disease associated with nephrotropic strains and a marked decrease in egg production. The virus can spread to different organs of the chicken (Boroomand *et al.,* 2012). Infectious bronchitis is a disease that causes economic losses in the poultry industry throughout the world (Cavanagh *et al.,* 2009; Boroomand *et al.,* 2012).

2.2 History

Infectious bronchitis was first described in 1931 in a flock of young chickens in the USA (Johnson and Marquardt, 1975). Since that time, the disease has been identified in broilers, layers and breeders throughout the world (Johnson and Marquardt, 1975; Gary *et al.*, 2009). It has been reported in the continents of America (Johnson and Marquardt, 1975), Europe (Gough *et al.*, 1992; Cavanagh and Davis, 1993; Capua *et al.*, 1994), Asia (Wang *et al.*, 1997) and Australia (Lohr, 1976; Ignjatovic and McWaters, 1991). In Africa, IB virus has been reported in Morocco in 1982–1983 (el Houadfi and Jones, 1985) and in Egypt in 2003 (Abdel-Moneim *et al.*, 2006). Antibodies against the virus were reported from Zimbabwe (Kelly *et al.*, 1994), South Africa (Thekisoe *et al.*, 2003), Botswana (Mushi *et al.*, 2006) and Nigeria (Komolafe *et al*.,1990; Ducatez *et al.*, 2004; Owoade *et al.*, 2006; Emikpe *et al.*, 2010; Mungadi *et al.*, 2015). Vaccines to help reduce losses in chickens were first used in the 1950s (Gary *et al.*, 2009).

The first serological evidence for the prevalence of IBV in West Africa was in Eastern Nigeria early in the 1990s by Komolafe *et al*. (1990). Also among the early reports of serological evidence of infectious bronchitis virus (IBV) was after a study conducted by Owoade *et al.* (2004), where serum samples of 52 flocks from poultry farms in Nigeria were tested for the presence of infectious bronchitis virus (IBV) antibodies using a commercial ELISA kit. Samples were collected from 1 to 7 day old chicks from 9 different breeders, 11 broiler, 19 pullet, 3 layer, 8 broiler breeder, 1 pullet breeder and 1 cockerel flocks. Forty seven out of the fifty two tested flocks (90%) were positive for IBV antibodies. Similar study was conducted by Owoade *et al.* (2006) in eight poultry farms in Nigeria; 1059 commercial chickens in the south-western part of the country were tested including chickens from nine breeder, 14 broiler, 28 pullet, 11 layer, and three cockerel flocks. They were tested for antibody

seroprevalence to the following poultry viruses of potential economic importance: infectious bronchitis virus (IBV), avian reovirus, avian pneumovirus (APV), infectious laryngotracheitis virus (ILTV), avian influenza virus (AIV), and avian leukosis virus (ALV). Seroprevalence of 84% were recorded for IBV.

Emikpe *et al.* (2010) also carried out a serosurvey in 672 chickens in southwestern Nigeria for antibodies to infectious bronchitis virus using ELISA. Samples were obtained from commercial and indigenous chickens of different ages in Oyo, Ogun, Ondo and Lagos states and a seroprevalence of 82.7% was obtained. Mungadi *et al.* (2015) carried out serological survey for IBV antibodies in local and commercial chickens in Sokoto state, and obtained a seroprevalence of 89%. Mungadi *et al.* (2015) recorded high prevalence of about 89% of IBV in Sokoto, north western Nigeria after testing 400 chickens using ELISA.

Characterization of strains of IBV was first evidenced in Nigeria by Ducatez *et al*. (2009) when QX-like IBV was reported from the backyard poultry in northern and southern parts of the country designated IBADAN strain (NGA/A116E7/2006), with unusual characteristics suggesting that it is a distinct variant unique to Nigeria.

2.3 Etiology

Infectious bronchitis is caused by infectious bronchitis virus (IBV) a coronavirus (Worthington *et al.,* 2008; Gary *et al.,* 2009; Zahra *et al.,* 2012).

2.3.1 Classification and Virus Characteristics

Infectious Bronchitis Virus (IBV), a corona-like appearance-‘crown virus’, is an enveloped, pleomorphic virus with a club-shaped projection about 20 nm in length, approximately 120 nm in diameter, single stranded ribonucleic acid (ssRNA) with

positive polarity (Jackwood, 2012). The virus is fairly labile and easily destroyed by disinfectants or organic solvents viz., ether and chloroform, sunlight, heat (56°C for 15 min), alkalis and other environmental factors (Otsuki *et al*., 1979).

Infectious Bronchitis Virus belongs to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Gammacoronavirus* and species *Avian corona virus* (ICTV, 2011). Among RNA viruses, the *Corona virus* has the largest genome which is linear non-segmented, positive-sense, single-stranded RNA approximately 27.6 kilo bases (kb) in length, (Kuo *et al*., 2013). IBV viral genome codes for four major structural proteins: the spike (S), membrane (M), the internal nucleoprotein (N) and small membrane (envelope) protein (E) (plate 2.1). Protective immunity, hemagglutination-inhibition (HI) and most of the virus neutralizing (VN) antibodies are induced by S1 protein that is formed by post translational cleavage of S protein. S1, the major antigen to which neutralizing antibodies develop, is the determinant of host species specificity and pathogenicity by determining susceptible cell range (tissue tropism) within a host. The N-terminal (S1) part of the S protein mediates attachment to cells via a receptor binding domain (RBD) and is the most variable part of the S protein having unique amino acid sequences determining the serotype. The spike glycoprotein of IBV is composed of two glycopeptides: S1 (90 kD) and S2 (84 kD). The S1 glycopeptide is found at the distal end of the spike protein and contains important epitopes that induce both virus-neutralizing and hemagglutining-inhibiting antibodies. S1 region varies greatly in its amino acid sequence and the variations are thought to be located within two hypervariable regions (HVRs) (Smati *et al*., 2002) (plate 2.1). Therefore, serotypic evolution in IBV seems to be associated primarily with the sequences of the S1 glycoprotein (Liu *et al*., 2006).

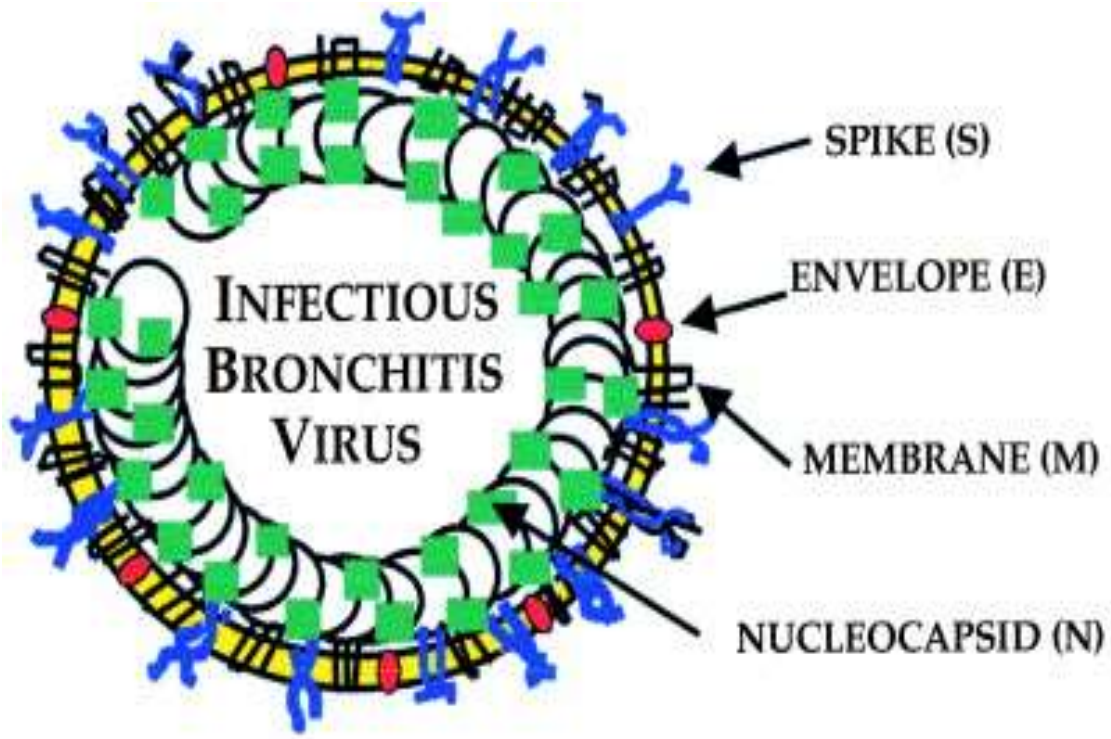
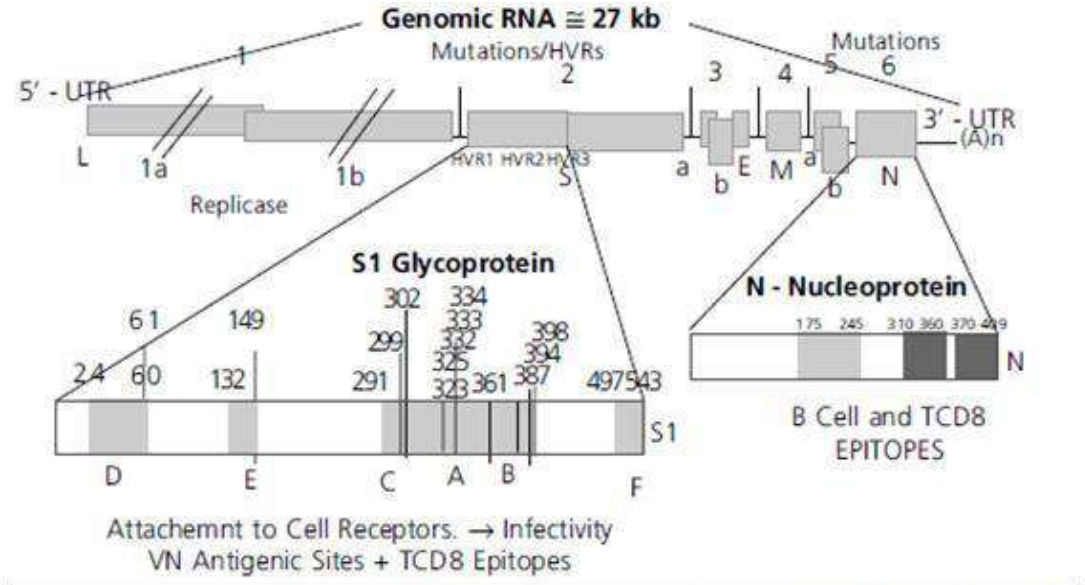


Plate 2.1: Structure of infectious bronchitis virus showing the structural proteins
Source: Ellen *et al.* (2000).



Genomic organization of IBV with mutation hot spots, their relationships with alterations in viral spike glycoprotein and nucleoprotein and the consequences on relevant biological and immunological properties of IBV.

Figure 2:1 Genomic organization of IBV with mutation hot spots

Source: Montassier (2010).

Classification systems of IBV strains are divided into two major groups: functional tests, which regard the biological function of the virus; and non-functional tests, which look at the viral genome. Typing by functional tests results in pathotypes, protectotypes and antigenic types (De Wit 2000; Cavanagh, 2005). The pathotype is defined by tissue tropism and predominant lesions displayed by an IBV strain in natural hosts (Cavanagh, 2005). Grouping of IBV strains into protectotypes provides direct data about the efficacy of a vaccine and strains that induce protection against each other belonging to the same protectotypes (plate 2.2). Antigenic typing characterizes the serotypes and epitope-types of an IBV strain, using virus-specific polyclonal or monoclonal antibodies respectively. The classification of IBV strains based on characterization of the genome results in genotypes. The main genotyping methods include nucleotide sequencing, detection of genotype-specific parts of the genome by RT-PCR, or determining the position of enzyme cleavage sites on a relevant gene by the restriction fragment length polymorphism technique (RFLP) (De Wit, 2000). To classify (genotyping) strains, S1-specific RT-PCR is followed by sequencing or restriction endonuclease analysis (Abdel-Moneim *et al*., 2006; Sumi *et al*., 2012).

There are several widely distributed classic and variant IBV genotypes (de Wit *et al.*, 2011). Wild type IBV isolates differ phenotypically from the parental vaccine strain (McKinley *et al.*, 2008; van Santen and Toro 2008; Gallardo *et al.*, 2010). Infectious bronchitis virus serotypes (old and new) differ from one another, as well as from vaccine strains by approximately 50% of S1 amino acid sequence (Casais *et al*., 2003). The C-terminal S2 part triggers fusion of the virus envelope with host cell membranes. Important mechanisms of viral variation and diversity largely depend upon the large size of RNA genome, permitting diverse means of mutations

and recombinations (Lai, 1996). At present, there is no definitive classification system. Serotypes are typed based on virus neutralization test (VNT) after isolating them in embryonated eggs or cell culture systems or in tracheal organ cultures (TOCs), whereas protectotype of a strain is typed based on cross-immunization study (CIS). Strains that induce protection against each other increase the efficacy of vaccination. Genotypes are grouped into strains based on genetic characterization using reverse-transcriptase-polymerase chain reaction (RT-PCR), followed by restriction endonuclease (RE) cleavage site analysis and sequencing (Callison *et al*., 2006). The virus has the ability to mutate or change its genetic makeup very quickly. Numerous serotypes of IBV have emerged which complicate control efforts through vaccination (Callison *et al*., 2006).

2.3.2 Replication Strategies

The multifactorial regulation of translation during an IBV infection can be due to the N protein causing down-regulation of host cell translation in IBV infected cells, with up regulation of the translation of virus-encoded proteins. The N protein may interfere with host cell translation by disrupting the formation of new ribosomes and possibly the cell cycle in the nucleolus, followed by binding to the 5' end of virus-derived RNA (Nelson *et al*., 2000). This causes recruitment of ribosomes for translation of viral RNAs. Alternatively, interaction of ribosomes with the *Corona virus* core can cause destabilization and release of genomic RNA thereby causing trafficking of virus particles to the nucleolus in association with ribosomal proteins (Hiscox and Ball, 1997).

2.3.3 Evolution of IBV

Even though it is not clearly known how coronaviruses, particularly IBV evolve, it is postulated that this involves one or more of the following: (i) mutation from nucleotide insertions, deletions, or point mutations as a result of polymerase proof-reading activity; (ii) genomic recombination between vaccines and field strains, which leads to multiple template switches as typically observed in the more virulent CK/CH/2010/JT-1 IBV isolate that originated from recombination of QX-like, CK/CH/LSC/99I-, tl/CH/LDT3/03- and 4/91-type IBV (Nix *et al.*, 2000; Zhou *et al.*, 2017). IBV genome analysis showed that regions encoding non-structural proteins 2, 3, and 16, and the S1 glycoprotein have the highest degree of diversity (Thor *et al.*, 2011); and (iii) viral selection pressure that may result from vaccination and presence of partially immune birds.

Changes in tissue tropism has also been reported to cause alterations in the coding sequences of several coronaviruses (Kuo and Masters, 2002; Read *et al.*, 2015). The alteration in the S1 amino acid sequence could occur during adaptation of IBV in Vero cells or following several passaging in chicken embryo (Fang *et al.*, 2005; Ammayappan *et al.*, 2009). Ultimately, viruses that are not ‘fit’ are eliminated, leaving only ‘fit’ ones to strive, spread, and cause devastating disease (Zhao *et al.*, 2017).

Plate 2.2: Protectotypes of IBV at electron microscopy

Source: Worthington *et al.* (2008).

The evolution of IB virus is constantly being reported due to intensive poultry farming practices, rapidly growing poultry industry, increased global trading and immense pressure of vaccination (Abro *et al*., 2012). New variants of IBV have emerged due to spontaneous mutation and recombination during virus replication, followed by replication of those phenotypes favored by selection (Abdel-Moneim *et al*., 2012; He *et al*., 2012; Toro *et al*., 2012a, b; Liu *et al*., 2013b; Mo *et al*., 2013) and cause significant disease in vaccinated flocks of all ages. It is assumed that due to widespread vaccination, the immune selection pressure involving the S1 subunit of S gene and the high mutation rate of the viral genome together might have resulted in the emergence of many serotypes and variants (Abro *et al*., 2012). Existing IB vaccines do not provide protection against these (Kuo *et al*., 2010). New live-attenuated vaccines are usually required mainly based on locally prevalent field strains. Important IBV variants are: D274 and D1466 (Netherlands), B1648 (Belgium), AZ20/97 (Italy), Arkansas (USA), 84084 and 88121(France), 4/91(UK). Different environmental determinants within the host viz., immune responses, affinity for cell receptors, physical and biochemical conditions are also implicated in the selection process (Toro *et al*., 2012a). Recombinations in IBV S1 and N genes have been documented in several field isolates (Cavanagh, 2007; Kuo *et al*., 2010, 2013; Abdel-Moneim *et al*., 2012; Liu *et al*., 2013a). Variants may attain increased virulence, efficient receptor binding, rapid transmission and survival in host system. It is important to isolate and type the IBV virus strains prevailing in a geographical area periodically and vaccinate birds accordingly with vaccine strains that offer maximum cross protection against the field virus in question or use combination of different strains to provide broad protection. Similarly, vaccines could be developed from recently isolated IBV field strain(s) from particular regions. Amino acid substitutions

in epitopes responsible for development of neutralizing antibodies can result in immune evasion (Shi *et al*., 2006). Thus, nucleotide sequencing and identification of amino acid substitutions especially involving S1 and N gene help to identify vaccine failure due to antigenic variation (Kuo *et al*., 2013). Variants are generally related to the appearance of positively selected, single point mutations or recombination in the antigenic domain of the viral proteins which occurs continuously (Lee *et al.*, 2001; Jackwood *et al*., 2005; Kuo *et al*., 2013). These mutations lead to the alteration of virulence and the escape of the viruses from host defenses (Lee and Jackwood, 2001). The average synonymous mutation rate in all coronaviruses including IBV is approximately $1.2x10^{-3}$ substitutions/site/year (Hanada *et al*., 2004; Holmes, 2009). The use of modified live vaccines has helped in rapid evolutionary rate of IBVs. Understanding the evolution of IBV is essential with regard to development of control and prevention strategies (Jackwood *et al*., 2012).

2.3.4 Classification of Strains

Serotyping by virus neutralization (VN) and haemagglutination inhibition (HI) and genotyping are the common methods of classification of IBV strains. Serotype specific monoclonal antibodies are induced by S protein (S1 subunit) but are available for a few serotypes in a small quantity. New serotype may emerge due to mutation. Nowadays, to classify (genotyping) strains, S1-specific RT-PCR is followed by sequencing or restriction endonuclease analysis (Abdel-Moneim *et al*., 2006; Sumi *et al*., 2012). Deduced S1 amino acid sequences with virus neutralization (VN) tests have revealed many serotypes. These serotypes differ by about 20-25% (amino acid), or by 40% or more occasionally, example being 7.6% difference between Conn 46 and Mass 41 strains in the S1 region or amino acid identity (97%) with D274.4

clusters, grouped into 7 serotypes based on RT-PCR analysis of the N gene and neutralization tests, respectively (Roussan *et al*., 2009).

In North America, Massachusetts, Connecticut and Arkansas 99 IB viruses are common serotypes. A large number of variant viruses (82) have been reported, however only GAV and GA98 were found to be implicated in widespread disease disseminations and persistent virus infections. Other IBV variants involved were Mass, Conn, Ark-DPI, CAV, DE072, MX97-8147, etc. (Jackwood *et al*., 2005). Three variants viz., CA557/03, CA706/03 and CA1737/04 were not related to each other or to Conn, Ark, or Mass vaccine strains, genetically or by cross-virus neutralization test (Kingham *et al*., 2000; Ziegler *et al*., 2002; Jackwood *et al*., 2007). Most of the Brazilian IBV field isolates recorded up to 1989 were classified as Mass (Massachusetts) serotype. Seroprevalence of the IBV in various species of birds in some of the countries (like Grenada) was reported, although vaccination against IBV is not practiced (Gutierrez-Ruiz, 2004; Sabarinath *et al*., 2011; Ramirez-Gonzalez *et al*., 2012). There are five distinct genotypes: A, B, C, D and Massachusetts based on the RFLP patterns of twelve Brazilian isolates and one reference vaccine strain (De Fatima *et al*., 2008; Rimondi *et al*., 2009; Acevedo *et al*., 2012). Massachusetts-serotype vaccine is the only one type of live attenuated vaccine approved in Brazil, but the genotypes are greatly divergent and most of them belong to Non-Massachusetts types (Di Fabio *et al*., 2000; Villarreal *et al*., 2007; Brandao, 2010). Interestingly, in Argentina, putative genotypes have been observed (De Fatima *et al*., 2008; Rimondi *et al*., 2009; Acevedo *et al*., 2012). The Massachusetts type was first reported in 1948 in UK and subsequently in Holland new serotypes were reported, against which vaccines were developed (Cook *et al*., 1996).

The prevalence of IBV strains and the disease in the Middle East varied from country to country. A Chinese-like recombinant virus (DY12-2-like) was reported for the first time in the Middle East (Seger *et al.*, 2016).

From Initial reports in Iran, the Mass-like IBVs are the most commonly isolated serotypes (12 isolates), followed by the European D274 and 4/91 (793/B)-like strains (3/2001 and 14/2001) (Mayahi and Charkhkar 2002). Subsequently, it was shown that the 4/91-like is more prevalent in broiler chickens than the Mass type serotypes (Bande *et al*., 2017).

The Iranian IRFIBV32 variant, 793/B or CR88-like serotype, has wide tissue distribution, causing marked lesions in the respiratory, urogenital, and digestive systems (Boroomand *et al.*, 2012). The strains were also shown to exhibit tropism for the bursa of Fabricius, as observed following inoculation with Iranian IR/773/2001. This suggests that the IRFIBV32 variants have immunosuppressive potential (Mahdavi *et al.*, 2007). The Iranian IBV isolates were also characterized by S1 gene sequencing, and these isolates were then grouped into six-distinct phylogenetic clusters; namely, IS/1494/06 (Var2)-like, 4/91-like, QX-like, IS/720-like, Mass-like, and IR-1 (3%), with isolation rates of 32, 21, 10, 8, 4, and 3%, respectively (Najafi *et al.*, 2006).

The 4/91 IBV serotype is prevalent in Iraq. There are vaccines available for this and the Ma5 and H120 serotypes. A novel IBV variant, the Sul/01/09, is also prevalent in Iraqi broiler farms, which is distinct from the vaccine and other serotypes reported in Iraq and neighboring countries (Mahmood *et al.*, 2011). Between 2014 and 2015, four major groups were reported in Iraq; namely, group I: variant 2 [IS/1494-like], group II: 793/B-like, group III: QX-like, and group IV: DY12-2-like genotypes. There were 96.42–100, 99.68–100, and 99.36–100% nucleotide sequence

identity within groups I, II, and III, respectively. Group I (variant 2) was the most commonly isolated IBV in Iraq.

In Jordan, the IBV strains identified include Ark, DE-072, and Mass (Gharaibeh, 2007). Other variants detected include 4/91 and D274 (Roussan *et al.*, 2008). Antibodies to M41, 4/91 and D274 were detected in clinically healthy flocks using serotype-specific antisera (Roussan *et al.*, 2009). Five QX-like IBVs, designated JOA2, JOA4, Saudi Arabia-like (Saudi-1, Saudi-2), and Iraq-like strains were also identified. Phylogenetic analysis showed that the five IBV isolates were 96.6–99.1% related to a Chinese QX-like strain, CK/CH/LDL/97I, and with <80% nucleotide similarity to the M41 and H120 vaccine serotypes. The CK/CH/LDL/97 strain was thought to be associated with sporadic IB outbreaks in the Middle East. The appearance of new IBV strains in Middle Eastern countries was postulated to be as a result of recombination between live attenuated vaccine viruses and field strains (Ababneh *et al.*, 2012).

Abdel-Moneim *et al.*, (2006) reported in Israel 13 new IBV variants out of which 11 are closely related to the previously reported Israel variant strains, IS/885 and IS/1494/06, and two isolates are clustered with the European CR/88121 and/or 4/91strains (Selim *et al.*, 2013).

In Pakistan, the prevalent IBV variants were M41, D-274, D-1466, and 4–91 based on antibody titers. Recently, a novel nephropathogenic IBV variant, PDRC/Pune/Ind/1/00, in Western India was molecularly characterized. This variant was isolated from commercial broiler chickens that manifested clinical signs such as visceral gout and severe nephrosis (Bayry *et al.*, 2005). Although IB vaccines are used in these countries, their effectiveness toward the local strains has not been evaluated (de Wit *et al.*, 2011).

An extensive epidemiological study on IB in Russia, Ukraine and Kazakhstan, between 2007 and 2010, showed the dynamics of IBV has changed with the Mass, 793/B, D274 and QX-like IBV, now becoming the most prevalent genotypes, followed by the B1648, Italy-02, and Arkansas variants. Eleven 4/91-related IBV isolates were reported, which included recombinants of the field and vaccine strains and the local strains designated UKR/02/2009 (or 4/91), RF/03/2010, and RF/01/2010 (Ovchinnikova *et al.*, 2011).

Variant IBV isolates were first reported in Europe in early 1970s (Dawson and Gough, 1971). In the UK, 793/B (also known as 4/91 and/or CR88) was identified as the predominant serotype (Cavanagh *et al.*, 1999). Other European serotypes of Mass IBV genotype were also identified in the UK (Gough *et al.*, 1992), France (Auvigne *et al.*, 2013), Belgium (Meulemans *et al.*, 2001), Italy (Capua *et al.*, 1994; Zanella *et al.*, 2003), Poland (Domańska-Blicharz *et al.*, 2007), and Spain (Dolz *et al.*, 2006, 2008). Of the European serotypes, 793/B, also known as 4/91 and CR88, D274 remained of international concern because of their propensity to spread within and outside Europe (Gough *et al.*, 1992; Abro *et al.*, 2012).

IBV was first reported in the mid-1980s in China. To control IBV infection in chickens in this country, the live attenuated and killed-oil adjuvant vaccines, derived from Mass (H120 and Ma5) and Conn serotypes, were used. However, these vaccines only served to reduce, not eradicate, the problem, because the disease continued to remain a major threat to the poultry industry (Han *et al.*, 2011). The Chinese isolates were separated into five genetic groups (genotypes) viz. (LX4, 4/91, JP, Gray and Mass) and seven serotypes based on *S1* HVR I nucleotide sequences and virus-neutralization test, respectively (Chen *et al*., 2009; Li *et al*., 2012). Thirteen isolates belong to serotype I; two isolates, GX-NN10 and GX-YL2, vaccine strains H120 and

Ma5 and one reference strain, M41, belong to serotype II; five isolates belong to serotype III; isolate GX-YL1 belongs to serotype IV; isolates GX-GL1 and GX-NN7 as well as one vaccine strain, 4/91, belong to serotype V; isolates GX-YL8 and GX-YL9 belong to serotype VI. Neutralization titers of isolate GX-NN12 were relatively low against all the antisera, so it belongs to serotype VII (Wang *et al*., 1997; Wu *et al*., 1998; Li and Yang, 2001; Yu *et al*., 2001a; Liu and Kong, 2004). The QX and LX-like IBV strains were also isolated, which are distinct from known vaccine serotypes (Yudong *et al.*, 1998; Zhao *et al.*, 2017) and these strains are broadly classified as A2-like and QX-IBV strains (Xu *et al.*, 2007; Zou *et al.*, 2010; Li *et al.*, 2013). Yu, *et al*. (2012), reported a recombinant nephropathogenic IBV strain, IBV-YX10 in 12 days old broiler chicks in China.

Malaysia first documented cases of IBV infection in 1967. Most IBV isolated before the 1990s were antigenically similar to the vaccine strain viruses of the Mass serotype (Arshad, 1993). Subsequent studies identified two unique IBV variants, the nephropathogenic variant, MH5365/95, and the respiratory pathogenic strain, V9/04, isolated in 1995 and 2004, respectively. These variants were later shown to have remarkable similarity with several Chinese isolates (Zulperi *et al.*, 2009).

In Singapore, most serotypes identified, based on their antigenic relatedness, were classified under Mass-like serotype (Yu *et al.*, 2001).

IBV was first described in the 1970s In Indonesia (Ronohardjo, 1997). Based on antigenic characteristics, an Indonesian isolate, I-37, cross-reacted with the Conn 46 strain of US origin; three isolates, I-269, I-624, and PTS-II, cross-reacted with the Mass 41 vaccine strain, while two isolates, I-625 and PTS-III, were related to Australian N2/62 strain (Indriani, 2000). Further analysis of the I-37 isolate showed

differences of approximately 6.9 and 15.6% in nucleotide and amino acid sequences, respectively, with the Conn-46 isolate (Dharmayanti *et al.*, 2005).

In South Korea, most IBV variants are of nephropathogenic pathotypes, classified either as KM91-like, QX-like, or recombination strain (Lim *et al.*, 2011). A recent analysis of 27 IBV variants isolated from 1990 to 2011 classified the Korean IBV isolates into five genotypes: (i) Mass vaccine serotype, (ii) Korean-I (K-I), (iii) Chinese QX-strain-related, (iv) KM91-like isolates, and (v) isolates that do not fit into any known group of Korean strains. Two genotypes, 11036 and 11052, appeared to be generated from recombination events between the new Korean genotype in cluster 1 and Chinese QX-like strain and between K-I and H120-vaccine serotype, respectively (Mo *et al.*, 2013).

In Japan, local variants have shown a different clustering pattern from existing isolates but are closely related to isolates from China and Taiwan. Local isolates such as JP/Wakayama/2003, JP/Iwate/2005, and JP/Saitama/2006 from non-vaccinated flocks share identity with 4/91 variant, possibly of French or Spanish origin. On the other hand, one Japanese variant, JP/Wakayama-2/2004, isolated from 4/91-vaccinated flocks is related to the vaccine strain (Mase *et al.*, 2008; Shimazaki *et al.*, 2009).

In Thailand, analysis of *S1* HVR I nucleotide sequences separated the viruses into two groups (I and II) (Pohuang *et al*., 2009). The sequence analysis of the *S1* gene demonstrates that NIB Indian isolate PDRC/Pune/Ind/1/00 possesses a unique genotype compared to other reference strains of various countries and is unrelated to North American, European and Australian strains (Bayry *et al*., 2005). Recently, an Indian IBV strain (India/LKW/56/IVRI/08) revealed 99% homology with a Thailand strain (THA280252) and the other strain (India/NMK/72/IVRI/10) showed even

greater homology with IBV strains from UK (4/91 pathogenic strain), Japan (JP/Wakayama/2/2004) and China (TA03) (Sumi *et al*., 2012).

The disease known as uraemia was first recognized in Australia in the late 1940s. Cumming (1963) isolated Nl/62 virus (synonym 'T') from one such case in 1962, a prototype of nephropathogenic strains of IBV. They were found to differ antigenically from other IBV strains having no common epitopes on either the N or M proteins (Ignjatovic and McWaters, 1991). These findings have indicated that unusual changes are occurring in strains isolated in Australia. Infectious bronchitis virus strains viz., Ql/88, Ql/89, N3/88, N6/88, Nl/89, N2/89, N2/90, N5/90, Nl/94, N6/94, V18/91, V19/91, V6/92, V9/92, Vl/93, V2/93 and V3/93, shared only minor antigenic similarity with the classical infectious bronchitis virus strains and clearly belonged to group II of novel strains. There is no clear-cut correlation between the S1 amino acid sequences and the nephropathogenicity of strains (Ignjatovic and McWaters, 1991; Sapats *et al*., 1996). In New Zealand, IBV was first isolated in 1967 and four serotypes (A, B, C and D) of the virus were described in 1976 that were different from those present in other countries, based on virus neutralization tests. Subsequently, a vaccine was produced from one of those serotypes (A) which was said to protect against all serotypes and is currently used in layer and breeder flocks and are having similarity to Australian Vic S strain (Lohr, 1976; McFarlane and Verma, 2008). Cross protection offered by commonly employed vaccine strains against various known field strains has been described by Gelb *et al*. (1991).

A number of local variants are reported in Africa in addition to the widely known vaccine serotypes such as Mass and 4/91 strains (de Wit *et al.*, 2011a)., The IBV-G serotype was identified as a unique African variant In the late 1980s with

tropism to gastrointestinal tract. However, recent studies identified several other local non-vaccine types, including the QX-like strains and Italy 02, originally localized in China and Europe, respectively (Bande *et al*., 2017).

In Morocco, five different isolates were identified and designated as D, E, F, H, and M, and classified as the Mass serotypes. However, one isolate, IB-G, was found to be antigenically different from the five isolates and is unique to Morocco, this isolate has tropism for gastrointestinal tissues instead of the respiratory tract. Vaccine efficacy studies showed that immunization of chickens with a Mass-serotype vaccine, such as H120, only protected against challenge with IBV-E and -F and not -G (Ambali, 1992). RT-PCR and RFLP analyses on several samples following an outbreak of IB, in birds showing signs typical to that caused by the nephropathogenic strains revealed Mass serotype and those that were unique to Morocco (Al arabi, 2004). In January 2010 and December 2013, other IBV variants, including the IBV/Morocco/01 IBV/Morocco/30, and IBV/Morocco/38, were isolated in southern and central regions of Morocco (Dolz *et al.*, 2006, 2012; Fellahi *et al.*, 2015a, b).

Recent studies conducted in Eastern Libya showed the presence of 12 IBV strains that are phylogenetically classified in two distinctive clusters. Some of the isolates clustered together showed relatedness to the Egyptian IBV strains, CK/Eg/BSU-2/2011, CK/Eg/BSU-3/2011, and Eg/1212B. Other isolates from another cluster had relatedness to Egyptian Eg/CLEVB-2/IBV/012 and Israeli IS/1494/06 strains (Awad *et al.*, 2014). While the Eg/CLEVB-2/IBV/012 strain was reported to cause respiratory and renal pathology (Abdel-Moneim *et al.*, 2012), the IS/1494/06 strain can cause severe acute renal disorder (Meir *et al.*, 2004).

In Tunisia reported IBV variants, are TN20/00, TN200/01, and TN335/01. These isolates were phylogenetically classified under the same cluster as the CR88

(IB 4/91) and D274 isolates which were suggested to be associated with severe clinical disease and losses to the Tunisian poultry industry (Bourogâa *et al.*, 2009). Other variants reported between 2007 and 2010 were designated TN295/07, TN296/07, TN556/07, and TN557/07, were identified. These isolates were closely related to TN200/01, TN335/01, and Italy 02 variants, but distantly related to the H120 vaccine strains commonly used for poultry immunization in Tunisia (Bourogâa *et al.*, 2012).

In Algeria, Algeria28/b1, Algeria28/b2, and Algeria28/b3, were identified in chickens. These strains were determined as variants based on the S1 partial sequences. The pathogenic characteristics or immunogenicity of these genotypes have not yet been reported (Sid *et al.*, 2015).

Serological evidence of IB was first documented in Egypt in the 1950s (Ahmed, 1954). The disease continues to be a major problem in Egyptian poultry flocks In spite of efforts to control the infection using Mass vaccines. Local variant was discovered in 2002, designated Egypt/Beni-Suef/01 (Abdel-Moneim *et al.*, 2002). This isolate was found to be unique to Egypt but closely related to nephropathogenic strains, IS/1494/06 and IS/720/99 isolated in Israel (Meir *et al.*, 2004). Inoculation of the Egypt/Beni-Suef/01 IBV in chickens resulted in severe respiratory and renal diseases (Abdel-Moneim *et al.*, 2005). In 2006, another nephropathogenic variant, Egypt/F/03 closely related to the Dutch (D3128), Mass, and Israel IBV variants, were also identified (Abdel-Moneim *et al.*, 2006). In 2011, five other variants were identified, Ck/Eg/BSU-1/2011, Ck/Eg/BSU-4/2011, Ck/Eg/BSU-5/2011 (which clustered with Egypt/Beni-Suef/01 and Israeli IS/1494/06) Ck/Eg/BSU-2/2011, and Ck/Eg/BSU-3/2011. The variants were distinct from any known Egyptian variants or vaccine serotypes (Abdel-Moneim *et al.*, 2012). Molecular characterization suggested

the presence of two distinct genotypes that were classified as the vaccine strain GI-1 genotype and the GI-23 genotype, a variant field strain. The variant genotype was subdivided Egy/var I and Egy/var II, which resembled Israeli variants IS/1494 and IS885 respectively (Bande *et al.*, 2017). The IBV-EG/1586CV-2015 emerged following recombination of two viruses from the variant groups, Egy/var I and Egy/var II, which also suggests the intra-genomic diversity of IBV, particularly in the GI-23 genotypes (Zanaty *et al.*, 2016b). Subsequent studies of pathogenicity, by comparison with the classical genotypes, showed that the Egyptian IBV variant has multiple heterogeneous origins and diverse pathogenicity (Zanaty *et al.*, 2016a).

In Sudan, four isolates were reported; M114/2000, K179/2000, and K158/2000 belonged to the European 4/91 subgroup, while K110/200 was closely related to the Mass vaccine serotype (Ballal *et al.*, 2005). In a recent study, the novel IBV variants, designated Ck/Sudan/AR251-15/2014 and Ck/Sudan/AR252-15/2014 with 97% identity between these isolates and the SLO/305/08 from Slovenia and Kr/D42/05 isolate from Korea based on NGS. Based on amino acid sequence, 95% similarity was observed between these isolates and the Kr/354/03 from Korea and RF/28/2011 from Russia. Analysis of the HVR 3 amino acid sequence showed 98% highest identity with two Italian strains, the ITA/90254/2005 and AZ-40/05. The overall phylogenetic relationship using the HVR 1-2 and HVR 3 nucleotide sequencing of the S1 gene of IBV Ck/Sudan/AR251-14/2014 and Ck/Sudan/AR252-14/2014 showed clustering pattern with QX and QX-like (Naguib *et al.*, 2016).

In East Africa, IB was only recently reported to be present in Ethiopia (Hutton *et al.*, 2016) in a study using serology and sequencing approaches to detect IBV isolates from a non-vaccinated institutional farm in Debre Zeit, Ethiopia. The virus was found to be of European 793B genotype, with 92–95% sequence identity

with the French isolate, FR-94047-94, and the virulent 4/91 assumed to be a field isolate since neither the Mass nor 4/91 IBV vaccine is commonly used in African farms (Cavanagh *et al.*, 2005).

In South of Africa, one IBV variant was described in South Africa in 1984; however, this variant has not been fully characterized (Morley and Thomson, 1984). It was recently discovered that the Mass IBV serotype is predominant in the country while some QX-like and 793/B genotypes, the CK/ZA/2034/99 and CK/ZA/2281/01, were also found to be present (Knoetze *et al.*, 2014). The MJT1 and MJT2 variants were reported in non-vaccinated indigenous chickens in the Beitbridge region, bordering Zimbabwe. These chickens presented clinical signs that are associated with digestive and respiratory systems. The MJT1 and MJT2 isolates showed 98.6% nucleotide sequence similarity with a QX-like IBV strain, QX L-1148, suggesting that QX-like variants are involved in IB outbreaks in South Africa (Toffan *et al.*, 2011, 2013).

Serological evidence for the prevalence of IBV in Eastern Nigeria was shown early in the 1990s (Komolafe *et al.*, 1990) from South West Nigeria in the 2000s (Owoade *et al.*, 2004, 2006), and from Sokoto State, northern Nigeria (Mungadi *et al*., 2015). Recently a QX-like IBV was reported from backyard poultry in northern and southern parts of the country by Ducatez *et al.* (2009). The QX-like IBV variant, designated IBADAN strain (NGA/A116E7/2006), has a nucleotide diversity of 9.7–16.4% with previously known IBV genotypes. The NGA/A116E7/2006 did not cross-react with IT02 strain from Italy or with vaccine strains such as M41, D274, Conn or 793/B serotypes. The NGA/A116E/2006 variant showed minimal reaction with a QX-like strain, ITA/90254/2005, suggesting that it is a distinct variant unique to Nigeria (Ducatez *et al.*, 2009).

2.4 Host Range, Age Susceptibility and Tissue Tropism

The domestic chicken has been widely regarded as the exclusive host for IBV. The host range of IBV has been enlarging. Infectious bronchitis virus is also reported in pheasants, racing pigeons, peacocks, partridges and mallards and associated with respiratory disease, egg production and shell quality disturbances. Infectious bronchitis virus grows well in 9-12 days old embryonated chicken eggs via intra-allantoic route, producing curling and dwarfing of embryos. Infectious bronchitis virus has been propagated in chicken embryo tissue (kidney, lungs, liver), embryonic turkey kidney, Vero cells and chicken tracheal organ culture (Meir *et al*., 2004). Among all the age groups of birds, the young chicks are most ill affected and resistance develops according to age. Female chicks may harbor certain genotypes of the virus in the oviductal epithelium due to lack of maternal antibodies and such hens are termed as 'false layers'; the phenomenon being recently observed with a new variant (QX) predominant in Europe (De wit *et al*., 2011). Age-related susceptibility to infections is suspected to be under the control of an immunological response which in chicks develops fully at 2-5 weeks of age. Serotypes Massachusetts and Connecticut show more affinity for respiratory tract, whereas serotypes Holte, Gray and Australian 'T' strain are associated with nephrosis (Capua *et al*., 1999; Ignjatovic *et al*., 2002). Under certain circumstances, IBV is thought to persist for a considerable time in infected birds at some privileged sites like kidney and caecal tonsils (Almeida *et al*., 2012). Table 2.1 shows some of the genes contained in IBV genome, and table 2.2 shows strains of IBV prevalent in different continents.

2.5 Epidemiology and Economic Importance

Infectious bronchitis is prevalent in intensive poultry production system, having a high incidence of infection with significant economic losses to the world poultry sector, despite proper vaccination (Kuldeep *et al.*, 2014).

Reports regarding IB have since been given from different parts of the globe in several poultry raising areas caused by unique strains in different years (table 2.2, fig.2.2). More than 30 serotypes and dozens of virulent variants have also evolved (Zanella *et al*., 2003). The incidence of nephrosis that is caused by variants of IBV is on the increase in many countries which necessitates the development of new vaccines. Outbreaks of IB are often due to infections with the strains serologically different from the strains used as vaccines. Outbreaks mainly occur during winter (Lopez and McFarlane, 2006). The seasonal use of live virus vaccine (Ark) has led a subpopulation of the vaccine to revert back to virulence. Losses from production inefficiencies are more than from mortality. In broilers, IB leads to poor weight gain and loss of profit at slaughter. In layers, IB causes loss of egg qualities and egg production may drop down to 10-50%. Nephropathogenic strains cause mortality of up to 30% in susceptible flocks. In United Kingdom (U.K.), IBV is the biggest single cause of infectious disease-related economic loss (Pennycott, 2000; Meulemans *et al*., 2001).

Table 2.1: Some of the Genes Contained in 27.6kb IBV Genome

Gene	Properties and functions
Gene 1	Encodes two partially over lapping open reading frames (ORF) 1a and 1b.
Gene 2	Consists of one ORF that encodes the spike glycoprotein (S) which is post translationally cleaved into the amino acid terminal S1 (92-kDa) and the carboxyl terminal S2 (84-kDa) sub-units. The S1 sub-unit is involved in virus entry and also contains epitopes for virus neutralizing and hemagglutination-inhibiting anti bodies.
Gene 3	Contains 3 ORFs, 3a (174 nucleotides), 3b (195 nucleotides) and 3c (321 nucleotides). ORF 3c encodes the E protein which is a structural protein required for virion assembly while 3a and 3b encode structural proteins of unknown function. 3a and 3b are not essential for IBV replication in vitro thus considered to be accessory proteins.
Gene 4	Contains one ORF encoding the M glycoprotein, which is essential for the production of Corona virus like particles.
Gene 5	Present in all group 3 Corona viruses characterised to date. It is dispensable for replication in vitro using reverse genetics.
Gene 6	Has one ORF encoding the N protein which together with the genomic RNA forms the helical nucleocapsid. It is a highly conserved protein, has untranslated region (UTR) thought to be important for the initiation of negative-strand RNA synthesis.

Sources: Casai *et al*., (2005); de Haan and Rottier, (2005).

Table 2.2: List of the Strains of IBV Prevalent in Different Continents across the World

Continent	Prevalent strains
America	Mass, Conn, Florida, Clark333, Arkansas, GAV, GA98, CAV, MX97-8147, DE072, Gray, Holte, Iowa, JMK, CA557/03, CA706/03, CA1737/04, PA/Wolgemuth/98, PA/171/99, PA/1220/98
Latin America	Mass, Conn, Ark, 4/91(793 B), D274, SIN6, UADY, Cuba/La Habana/CB19/2009, Cuba/La Habana/CB6/2009, 50/96-Brazil, Chile 14, 22/97-Honduras
Europe	Mass, Italy 02, QX (D388), H120 793B (4/91 or Cr88), D207, D212, D3128, D3896, H52, H120, D387, V1385, V1397, D274, D212, D1466, D3128, D3896, D274, D1466
Africa	Mass, H120, IBADAN, TN20/00, TN200/01, TN335/01, TN295/07, TN296/07, TN556/07, TN557/07, Egypt/Beni-Seuf/01
Asia	Mass, H120, Ark, Gray, Ark99, CU-T2, Vic S, DE-072, JMK, D274, 793/B, IS/222/96, IS/251/96, IS/64714/96, IS/223/96, IS/572/98, IS/585/98, IS/589/98, IS/885/00, Egypt/Beni-Seuf/01, Sul/01/09, JP, SAIBK, LDT3, QX, KM91, K2, LX4, GX-G, GX-XD, LD3, LS2, LX4, LH2, LHI10, THA20151, THA40151, THA50151, THA60151, THA90151, PDRC/ Pune/Ind/1/00, RF/04/98, RF/07/98, RF/08/98, RF/10/98, RF/11/98, RF/12/98, RF/16/98, RF/17/98, RF/21/98, RF/01/99, RF/02/99, RF/03/99, RF/04/99, RF/05/99, RF/06/99, RF/13/99, RF/15/99, RF/20/99, RF/02/00, RF/03/00, RF/04/00, RF/08/00, RF/12/01, RF/14/01, RF/02/02, RF/05/02, GX-NN09032, GX-YL5, DY07, CK/CH/SD09/005, TC07-2, Q1, YN
Australia	N1/62, Vic S, N1/88, Q3/88, Q1/88, Q1/89, N3/88, N6/88, N1/89, N2/89, N2/90, N5/90, N1/94, N6/94, V18/91, V19/91, V6/92, V9/92, V1/93, V2/93, V3/93, A, B, C, D

Source: Kuldeep *et al.* (2014).

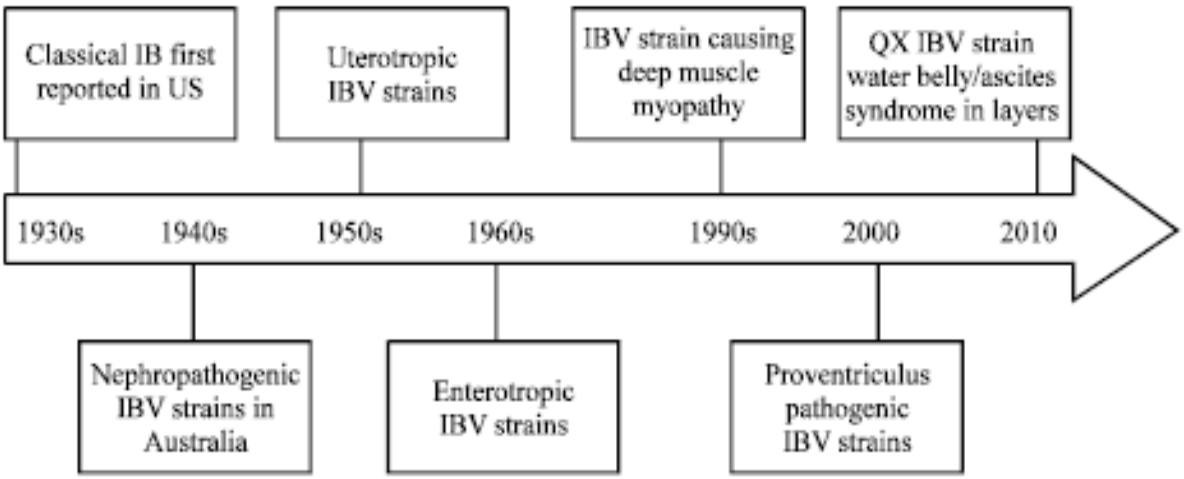


Figure 2.2: Chronological enlistment of emerging IBV stains isolated from different affected organs.

Source: Kuldeep *et al.* (2014).

2.6 Transmission and Spread

Infectious bronchitis is considered the most contagious poultry disease, and when it occurs, all susceptible birds in the premises become infected, regardless of sanitary or quarantine precautions (Anon, 2012). Coughing and sneezing are mainly responsible for spread of the virus in chicken flocks. A short incubation period of 18-36 hours is observed after IBV infection and thereafter clinical signs develop. Carrier birds rather than vectors are the nidus of infection. Disease can be transmitted through infected semen (Gallardo *et al*., 2011). Transmission from farm to farm is related to movement of contaminated people, equipment and vehicles. During an active outbreak air-borne spread of the virus over a considerable distance occurs. Following infection, chickens may remain carriers and shed the virus for several weeks (Gary *et al.*, 2009). A few birds may remain carriers and shedders of the virus for months after infection, they eliminate virus in secretions and discharges, thus exposing susceptible chickens or contaminating premises (Whiteman and Bicford, 1996). It is assumed that poultry industry in different parts of the world can contract the disease via migratory birds, even though the role of wild birds in the spread of IBV in most of the instances is speculative and largely unknown, deserving more attention and research activities (Sjaak de Wit *et al*., 2011).

The IB virus may persist on contaminated premises for approximately 4 weeks under favorable conditions and susceptible birds brought on the premises during that interval may contract the disease (Whiteman and Bicford, 1996).

2.7 Pathogenesis and Clinical Manifestations of the Disease

Infectious bronchitis virus has wide tissue tropism including respiratory, urogenital and digestive system and has pathogenic effects in tissues of respiratory tract, kidney and oviduct. Infection occurs via respiratory route regardless of tissue tropism of strains. Virus multiplies chiefly in the upper respiratory tract, following which viraemia occurs and the virus gets widely disseminated to other tissues. After the acute phase of infection, a persistent infection can be established in specific organs and the virus can be excreted continuously (Boroomand *et al*., 2012). The virus is primarily epitheliotropic and replicates in many epithelial cells, including those of respiratory tract, kidney and gonads producing lesions and in alimentary tract, many times with little pathobiological clinical effect (Ignjatovic *et al*., 2002). Infectious bronchitis virus infection of female chicks less than 2 weeks of age can cause permanent damage to reproductive tract (Ignjatovic *et al*., 2002).

Based on tissue tropism, there are two major IBV pathotypes, the respiratory and nephropathogenic pathotypes. Most classic IBV, such as the Massachusetts (Mass) serotype, infects the respiratory tract. However, the nephropathogenic strains, which occurr mostly in Asia and Middle Eastern countries, infect and damage the kidneys. The Moroccan IBV-G reportedly shows tropism for the gastrointestinal tract

(GIT). The QX IBV, first isolated in China from the proventriculus (Benyeda *et al*., 2009), are now present in other parts of Asia, Europe, Middle East, and Africa; they show altered tissue tropism, infecting both the kidneys and reproductive tract, causing 'false layers syndrome' and high mortality (Beato *et al.*, 2005; Irvine *et al.*, 2010; de Wit *et al.,* 2011; Amin *et al.,* 2012; Ganapathy *et al.,* 2012; Naguib *et al.,* 2016).

Infectious bronchitis occurs in birds of all age groups, but severely affects chicks evincing malaise, depression and retarded growth. Young birds of less than 3 weeks of age are more susceptible than older ones. The age and immune level of the poultry flocks and the pathogenic potential of the causative viral strain affects the nature and disease severity (Lee *et al*., 2004). Signs include respiratory symptoms (table 2.3), decreased egg production due to permanent damage to the oviduct and deterioration in egg qualities like watery albumin, misshapened and soft-shelled eggs (tables 2.4 and 2.5). Recovery from the typical respiratory phase may occur in birds infected with nephropathogenic viruses which subsequently show signs of depression, ruffled feathers and wet droppings along with increased water intake. Mortality, as high as 25% or more, occurs in chicken above 6 weeks of age (Lee *et al*., 2004).

Respiratory tract shows serous, catarrhal, or caseous exudates in the nasal passages, sinuses and trachea. Caseous plugs may be found in the lower trachea or bronchi of young birds. Air sac affections are characterized by thickening and opacity. Focal areas of pneumonia may appear. In urogenital system, middle third of the oviduct is most severely affected and may be non patent and hypo-glandular, continuously patent but underdeveloped structure to a blind sac. The kidneys are swollen and pale with the tubules and ureters often distended with urates (table 2.6), microscopic changes of the kidneys may also be present (Abdel-Moneim *et al*., 2005). The abdominal cavity of chickens in production may contain yolk substances, a

condition also observed in diseases leading to marked fall in egg production (Ahmed *et al*., 2007). Pathology is not usually associated with infection of the alimentary tract by enterotropic IBV. Pathogenic strains cause thickened, haemorrhagic or ulcerative lesions in the proventriculus, haemorrhagic lesions in the caecal tonsils and thickening of duodenum (Escorcia *et al*., 2002).

2.8 Pathology

2.8.1 Gross Pathology

Lesions associated with IB include a mild to moderate inflammation of the upper respiratory tract. If complicating factors are present, airsacculitis and increased mortality may be noted, especially in younger chickens (Gary *et al.,* 2009). Severe airsacculitis is manifested as a marked thickening and opacity of the air sac membranes and often is accompanied by exudate in the air sacs; it can also result in high mortality in young and growing birds especially if husbandry is poor, older birds are usually more resistant (Whiteman and Bicford, 1996). Kidneys of affected chickens will be pale and swollen. Urate deposits may be observed in the kidney tissue and in the ureters, which may be occluded (Gary *et al.,* 2009). In pullets that had IB or a severe vaccination reaction while less than 2 week old, there may be abnormalities of the oviduct (particularly the middle third) in occasional birds, oviducts may be hypoplastic or cystic and such birds may deposit yolks or fully formed eggs in the abdominal cavity and are referred to as internal layers (Whiteman and Bicford, 1996). Affected embryos examined at 7 days after inoculation are stunted and have an excess of urates in the kidneys. The amnion and allantoic membranes are thickened and closely invest the embryo; these embryos do not hatch out (Anon, 2005).

Plate 2.3: Dullness (top) and respiratory distress (bottom) exhibited in chickens infected following experimental infection with IBV

Source: Bande *et al*. (2016).

Plate 2.4: Irregularity in the shape and sizes of eggs from natural IBV infected breeder chickens.

Source: Bande *et al*., (2016).

Plate 2.5 Watery albumen from IBV infected chicken (left) compared to normal egg (right).

Source: Bande *et al*., (2016).

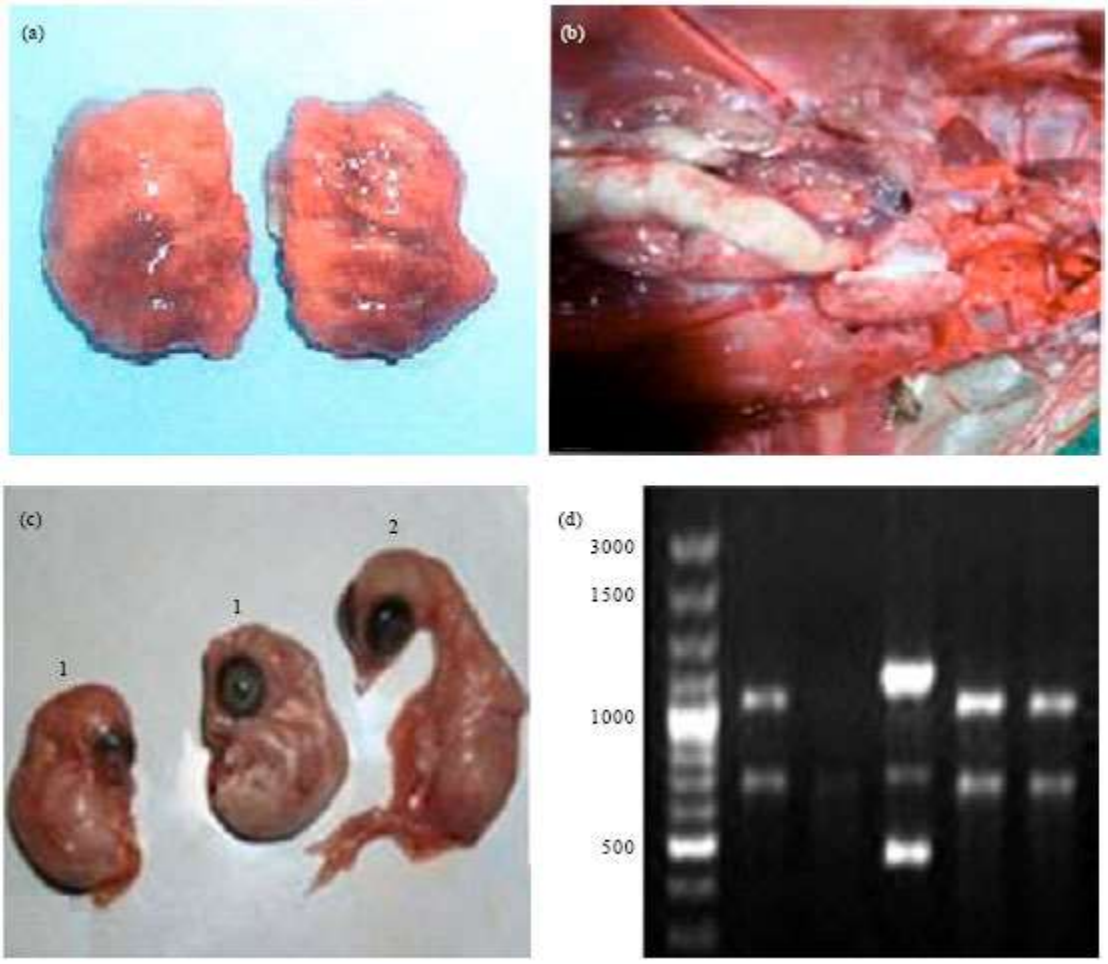


Plate 2.6: IBV Lesions and PCR Based Diagnosis

(a) Lungs of experimentally infected chicks showing areas of consolidation,

(b) Swollen ureter distended with urates in a chicken naturally infected with nephropathogenic IBV,

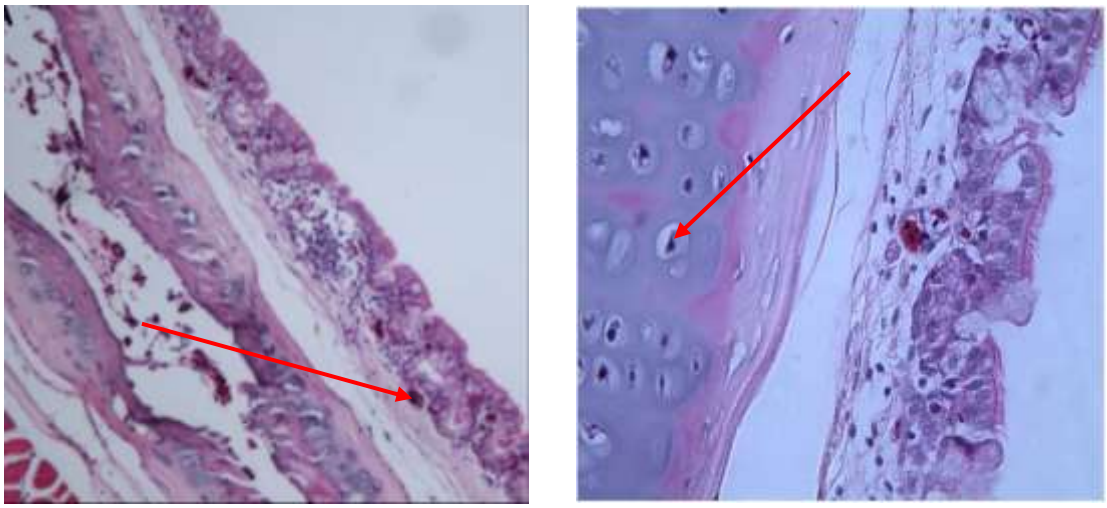
(c) IBV infected chicken embryos (1) Exhibiting dwarfing and curling compared with uninfected embryo (2) and

(d) Pattern of PCR-amplified S1 gene

Source: Worthington *et al.*, (2008).

2.8.2 Microscopic Lesions

In chickens suffering from IB, severe congestion of the lungs is seen (Abdel-Moneim *et al.,* 2006). Mucous, marked loss of cilia, desquamation, mononuclear cell infiltration, epithelial hyperplasia and vascular congestion of the trachea are also seen (table 2.7) (Abdel-Moneim *et al.,* 2006). Tracheal organ cultures (tracheal rings) may also be used for isolation of IB viruses. In this case, ciliostasis and damage to the tracheal epithelium are seen within 48-72 hours of inoculation, when the tracheal rings are observed under low power microscopy (Anon, 2005).Infection with nephropathogenic strains shows varying degree of pathological changes in the tubules with relatively unaffected glumeruli (Cavanagh and Gelb, 2008), foci of mononuclear cell infiltration are prominent in the cortex and medulla particularly in chronic nephritis (Cavanagh and Gelb, 2008). Cytoplasmic IBV immunoflourescence is found in all segments of the tubules, evidence of virus replication is found in the cells of tubules, and a large number of virus inclusion bodies are seen containing dark smooth particles within a single membrane (Chong, 1982). Kidney damage may be significant (Gary *et al.,* 2009). Microscopic lesions in the oviduct consist of areas of hypoplasia, non patent occlusion of oviduct lumen and development of cyst. Typical lesions in infected embryo that occur at about 5-7 days post inoculation are curling and dwarfing of the embryos, clubbing of downs, red or hemorrhagic embryos, and possibly white urate deposits in kidneys (Anon, 2005).



(a) (b)

Plate 2.7: Histopathological changes in the trachea of chicken naturally infected with IBV.

Note: (a) The marked infiltration of lymphocytes within the epithelia

(b) Evidence of mucosal secretions of goblet cells

Source: Bande *et al*. (2016).

2.9 Diagnosis

Infectious bronchitis can be diagnosed on the basis of clinical manifestation of disease, rising antibody titers and detection of antigen and viral DNA in the tissue sections and clinical materials (Chong and Apostolov, 1982).

Diagnosis can be made by recovering of the virus from the kidneys as well as feces of birds with acute and chronic nephritis (Chong and Apostolov, 1982). Infectious bronchitis virus can be isolated from the trachea, lungs and kidneys of infected chickens (Sylvester *et al*., 2003a; Dhama *et al*., 2011a, b) while liver and pancreas are primarily used for antigen detection; trachea and spleen for histological diagnosis (Fan *et al*., 2012). Two or three blind passages may be required for primary virus isolation which can be a cumbersome and time taking process. Therefore, embryos of specific pathogen free (SPF) chickens or their Tracheal Organ Cultures (TOCs) are preferred for isolation of IB virus. Further, direct detection methods in infected tissues include immunohistochemistry, or *in situ* hybridization (Nakamura *et al*., 1991; Chen *et al*., 1996). Confocal microscopy can be used to detect the viral N protein both from healthy and uninfected cells and also reveals the presence of this important protein (required in viral replication cycle) in the host cell (Hiscox *et al*., 2001). It is important to rapidly differentiate infectious bronchitis virus (IBV) from those of other disease agents like highly pathogenic avian influenza virus and exotic Newcastle virus, which can be extremely similar in the early stages of their pathogenesis (Callison *et al.,* 2010). High diagnostic accuracy can only be achieved by a series of laboratory assays (Villarreal, 2010). Laboratory tests to identify the viral genome, viral antigen (proteins) or antibodies against the virus are important to

confirm IBV infections (Worthington *et al.,* 2008). Testing serum samples at intervals (for example at the time of the clinical signs and 2 or 3 weeks later) provide the best basis for serological diagnosis; this is also applicable for monitoring vaccination results (Worthington *et al.,* 2008).

2.9.1 Field Diagnoses

These include clinical signs and postmortem lesions.

2.9.1.1 Diagnosis Based on Clinical Signs

Diagnosis of infectious bronchitis on the basis of clinical signs alone is very difficult (Worthington *et al.,* 2008). Diagnosis cannot be based solely on clinical signs because of similarities to mild respiratory forms of Newcastle disease, Infectious laryngotracheitis, and infectious coryza. Isolation or identification of the causative agent is required for a definitive diagnosis of IB (Gary *et al.,* 2009).

2.9.1.2 Diagnosis Based on Postmortem Lesions

Lesions in embryos are helpful in diagnosing IB (Gary *et al.,* 2009). The field IB virus may have to be serially passed in embryos to adapt the field virus to the embryos before typical lesions are recognized (Gary *et al.,* 2009; Anon, 2005). Post mortem findings are often not conclusive (Worthington *et al.,* 2008).

2.9.2. Laboratory Diagnosis

Infectious bronchitis can be diagnosed by detecting the virus itself (or parts of it) or by determining the specific antibody responses (Villarreal, 2010). The choice of the best test and its subsequent interpretation may be very confusing (Villarreal,

2010). Factors that influence the successful detection of IBV according to De wit (2000) are:

a) Time elapsed between the beginning of the infection and sampling: Upper respiratory tract is the primary site of IBV replication, which is followed by viraemia, causing the virus to disseminate to other tissues. All IBV types can be isolated from upper respiratory tract with the highest concentration in the trachea during the first three to five days post infection. After this period virus titer falls quickly in the second week below the detection levels. A complicating factor from the diagnostic point of view is the definition of which organ and how many birds are virus carriers (be it from vaccine or field origin). The two main sites of the virus persistence are cecal tonsils and kidneys.

b) Chicken immunity level at the time of infection: The level of acquired immunity at the time of infection has the highest influence on the period and quantity of IBV that can be detected. The presence of maternal antibodies does not reduce the level of re-isolation of challenge virus in the trachea and in the kidney after the challenge of two day old chicks.

c) Number of sampled chickens: Taking fewer samples than required decreases the chances of detecting IBV infection.

d) Selection of organs (samples): When acute breathing symptoms are predominant, the respiratory tract is the preferred site for sample collection. Kidneys, cecal tonsils and cloaca are sampled preferably when there are chronic infections or infections in vaccinated chickens.

e) Sample quality: The samples should be quickly refrigerated to preserve virus viability. If the freezing or refrigerating is not possible, samples should be placed into 50% glycerol, where IBV remains viable for many days.

f) Bird's genetics: Several studies indicate that genetic aspects may influence the susceptibility to IBV. Different types of chickens present variable mortality after the inoculation of IBV alone or in co-infection with E. coli.

Because the virus exhibits great antigenic variation, the serotype should be identified if possible; serotypes are conventionally identified with the aid of known serotype-specific chicken antisera in the virus neutralization test (Anon, 2005). However, the virus neutralization test is expensive, time consuming, and not readily available; therefore, it is not commonly used (Anon, 2005). Restriction fragment length polymorphism (RFLP) and reverse transcription polymerase chain reaction (RT-PCR) are two current methods commonly used for the detection of IBV and its serotype (Callison *et al.,* 2010). A rapid highly sensitive and specific method is needed in the differential diagnosis of different serotypes. A multiplex PCR method was developed and optimized to simultaneously detect Massachusetts (Mass) and Arkansas (Ark) serotypes of IBV (Ramneck and McFarlane, 2005).

Confirmation is made by nucleic acid-based methods: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using-specific oligonucleotide primers, producing DNA copies of the *S1* part of the spike glycoprotein gene to determine the identity of a field strain; restriction fragment length polymorphism (RFLP), RT-PCR and sequencing can identify all known serotypes of IBV as well as variant viruses (Meir *et al*., 1998; Sylvester *et al*., 2003b, 2006; Almeida *et al*., 2012; Jackwood *et al*., 2012; Sumi *et al*., 2012).

RNase T1 fingerprinting analysis can be used for fingerprinting of the IBV genome, advantage being use of complete genome for generation of strain-specific fingerprints (De Witt, 2000; Alvarado *et al*., 2005; Dhama *et al*., 2011b).

2.9.2.1 Diagnosis using Serology

Serological tests include group specific Enzyme Linked Immune-sorbent Assay (ELISA) (including monoclonal antibody based technique), Virus Neutralization Test (VNT) (type specific) and Haemagglutination Inhibition (HI), indirect Fluorescence Antibody Test (IFAT) and Agar Gel Immuno Diffusion (AGID) (Lin *et al*., 2012). Fluorescent Antibody Technique (FAT) is also a good test for antigen detection. Nucleocapsid phosphoprotein gene-specific Reverse Transcription Loop-mediated isothermal Amplification (RT-LAMP) assay has also been developed (Chen *et al*., 2010). However, for detection as well as serotyping, VNT is the gold standard test of choice while cross neutralization tests are used for detection of the variants. The fact that live IB vaccines are used globally and antibodies may fail to detect variant strains with modified epitopes, this is taken into consideration while developing tests for detecting antigen (Elankumaran *et al*., 1999; De Wit, 2000; Chen *et al*., 2010).

Some factors may influence the success of IBV antibody detection, such as age at the time of infection or vaccination, the degree of immune response may decrease when infection happens at very early stage, the presence of maternal antibodies at that time may delay or reduce serologic response (Villarreal, 2010).

Presence of immunity at the time of infection or vaccination; the sensitivity of test to detect antibodies may be much lower in vaccinated chickens as compared to non-vaccinated ones; number of sampled chickens; cross reaction between serotypes and occurrence of new and unexpected types of IBV (De wit, 2000).

i. Enzyme Linked Immunosorbent Assay (ELISA):

It is an immune enzymatic method and its automation enables the detection and titration of antibodies in a large number of serum samples (Villarreal, 2010). Most of the ELISA tests are generic for IBV, in other words, they do not differentiate serotypes, and this is due to the fact that the surface of the plate in which antigen-antibody reaction takes place is impregnated with the viral suspension in its complete form (Villarreal, 2010). The reaction is positive when any IBV strain is present. It detects IgG, and therefore, it is an indicator of humoral immunity, enabling the analysis of post-vaccination and infection responses in adult birds (Villarreal, 2010).

ii. Heamagglutination Inhibition test (HI):

Infectious bronchitis virus is not naturally heamagglutinating, and requires previous treatment with type C phospholipase enzyme to expose hemaglutinin which makes this test difficult to perform and to standardize (De wit, 2000). Hemagglutination-inhibiting antibodies are induced primarily against S1 spike protein and this test is serotype specific when used to detect antibodies after a single inoculation. HI specificity is much lower after IBV re-infection, especially when the second or subsequent serotype is heterologous (De wit, 2000).

2.9.2.2 Virus Isolation

Virus isolation can be laborious, time consuming and expensive (Villarreal, 2010). Additionally, the classic method of isolation may require several passages in embryonated egg until embryo mortality occurs or other signs are detected (Villarreal, 2010). Typically, this is done in specific pathogen-free chicken embryos at 9 to 11 days of incubation by the allantoic sac route of inoculation. Tissues collected for virus isolation attempts from diseased chickens include trachea, lungs, air sacs, kidney, and cecal tonsils (Worthington *et al.,* 2008). If samples are collected more than 1 week after infection, cecal tonsils and kidneys are the preferred sites for recovery of IB virus (Anon, 2005; Worthington *et al.,* 2008). Several blind passages may be necessary before clinical signs characteristics of IBV infections are observed in embryos (Anon, 2005).

2.9.3 Electron Microscopy

Electron microscopy provides a direct means of detecting and identifying IBV in biological samples based on morphological characteristics of Coronavirus. Positive cultures are confirmed based on the presence of Coronavirus-like pleomorphic structures with spike projections, following negative staining with phosphotungstic acid. Importantly, the shape and diameter (120 nm) of the virus are taken into consideration when making diagnostic judgments. Apart from the negative staining method, Transmission Electron Microscopy (TEM) is also a useful tool which enables the visualization of virus-like particles in infected cells (Arshad, 1993). However, this method is often applied to understand viral attachment and entry into the cell but is not a specific diagnostic test (Arshad *et al.,* 2002).

2.9.4 Immunohistochemistry

Immunoperoxidase and immunofluorescence are two important histochemistry methods for detection and confirmation of IBV antigen from infected tissue and/or cells. These methods work based on antigen-antibody reactions (Bezuidenhout *et al*., 2011). Immunoperoxidase methods such as the avidin-biotin complex (ABC) have been used successfully to localize IBV antigen in tissue samples (Abdel-Moneim *et al*., 2009). Likewise, indirect immunofluorescent assay is the most frequently used fluorescent technique (Abdel-Moneim *et al*., 2009).

2.9.5 Diagnosis by Molecular Assays

Virus typing has traditionally been performed by neutralization using selected IB antisera (Callison *et al.*, 2010). More recently, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) have been used to differentiate IBV serotypes (Callison *et al.*, 2010).

i. Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RT-PCR is found to be more sensitive than the IFA assay especially when more than one strain of IBV is involved, but the IFA assay is rapid and cheaper than the RT-PCR which exhibits higher sensitivity than virus isolation and could be used for rapid diagnosis of IBV in the field (Ramneck and McFarlane, 2005). It was gathered that real time PCR is more sensitive by 10 folds than virus isolation and 30 or more fold than by N gene or S1 gene RT-PCR respectively. RT-PCR targeting the N gene is more sensitive than common diagnostic assays allowing rapid and accurate IBV detection directly from clinical specimens, facilitating differential diagnosis (Callison *et al.*, 2010).

Reverse transcription-polymerase chain reaction product is digested with a set of specific restriction endonucleases; the generated fragments are separated by gel

electrophoresis and the specific pattern of their separation in the gel is compared with those of the standard strains for identification. Genotype identification is achieved by sequencing of the S protein (S1 subunit) gene. In US and Israel, RFLP analysis and RT-PCR product cycle sequencing are being used to identify field strains (Zwaagstra *et al*., 1992). Recently, the improvements in the PCR technique have created way for the application of real time-PCR for very rapid detection and quantification of virus (Jackwood *et al*., 2003); Multiplex-PCR and Multiplex nested RT-PCR technique for differential diagnosis of IB infection (Kataria *et al*., 2005; Dhama and Mahendran, 2008; Chen and Wang, 2010; Nguyen *et al*., 2013).

ii. Restriction Fragment Length Polymorphism (RFLP):

It is a molecular method of genetic analysis that allows individuals to be identified based on unique pattern of restriction enzyme (Subha *et al.,* 2010). RFLP is one technique used by forensic scientists in DNA fingerprinting (Subha *et al.,* 2010). Korean IBV field isolates were differentiated from non Korean IBV strains showing various genetic differences by amplifying the S1 glycoprotein gene of IBV using RT-PCR and analyzed by RFLP analysis (Lee *et al.,* 2004). RFLP allows differentiation of various known IBV strains, based on their unique electrophoresis banding patterns defined by restriction enzyme digestion (Kwon *et al.,* 1993). The assay was found to be comparable with traditional virus neutralization assay, although some strains such as the Gray and JMK strains were reportedly difficult to differentiate using arrays of restriction enzymes, thus limiting the universal application of this method (Kwon *et al.,* 1993).

iii. Multiplex Polymerase Chain Reaction (PCR):

To overcome the disadvantage of cost and to improve the diagnostic capacity of PCR, Multiplex PCR, a variant of the test in which more than one target sequence is amplified using more than one pair of primers has been developed (Elfath *et al.*, 2000). Multiplex PCR is used to detect viral, bacterial and other infectious agents in one reaction tool have been described (Elfath, 2000). The technique has been used in the diagnosis of infectious agents especially those which target viral nucleic acids (Elfath *et al.*, 2000). A Multiplex PCR was developed to amplify the S1 genes of vaccine and recent Taiwanese isolates of IBV (Hung *et al.*, 2003). A Multiplex PCR was also developed and optimized to detect six avian respiratory pathogens including six sets of specific oligo-nucleotide primers for IBV (Yaoshan *et al.*, 2002).

iv Sequencing and Phylogenetic Analyses

For genotyping, *S1* gene is usually amplified using RT-PCR, sequenced, and subjected to bioinformatics analyses (Zulperi *et al.*, 2009; Abro *et al.*, 2012). Following *S1* gene sequencing, isolates are characterized through bioinformatics analyses based on their phylogenetic relatedness with reference sequences available in sequence databases such as the National Center for Biotechnology Information (NCBI), EMBL, and DDBJ. Lack of standardization method among laboratories, particularly with respect to the *S1* gene segment length that is used in phylogenetic analysis, limits genotyping to some extent. Currently, molecular methods such as next generation sequencing (NGS) have been introduced to sequence whole genomes within limited periods of time (Bande *et al*., 2016).

2.10 Differential Diagnosis

Infectious bronchitis may resemble other acute respiratory diseases viz., Newcastle disease (ND), infectious laryngotracheitis (ILT), infectious coryza (IC) and

other none respiratory diseases like egg drop syndrome (EDS) that occur more severely and cause greater production losses when compared to IB. Moreover, nervous symptoms of ND, slow nature of spread of ILT, facial swelling of infectious coryza and ND are absent in case of IB. Production declines and shell quality problems in flocks infected with the EDS are similar to those seen with IB, except that internal egg quality is not affected in case of EDS (Cavanagh and Gelb Jr., 2008). Nutritional deficiency disorders such as vitamin A deficiency must also be taken into consideration while diagnosing IBV (Sylvester *et al*., 2005; Cavanagh and Gelb Jr., 2008).

2.11 Treatment, Prevention and Control

2.11.1 Treatment

There is no treatment for this disease (Anon, 2005; Anon, 2012). In young chickens it is helpful to increase the brooder temperature and provide as nearly ideal environmental conditions as possible (Anon, 2012). Sodium salicylate 1g/litre has been used as analgesic in acute phase of the disease; antibiotics to control secondary bacterial infections like colibacillosis are also used (Paul, 2004).

The entire flock should be treated for secondary bacterial infections with a water-soluble broad-spectrum antibiotic for 7-10 days (Anon, 2012). Tetracyclines and chlortetracycline may be effective for this purpose (Anon, 2012). If there is a particularly valuable bird that is showing upper respiratory symptoms, then treatment with a broad-spectrum injectable antibiotic may be more effective (Anon, 2012).The full course of treatment should be run through, as the affected birds usually show

marked improvement after just three days of treatment. If the birds are only showing a droopy posture with no other symptoms, then they should not be subjected to antibiotic treatment since it cannot be processed properly if the birds are suffering from kidney failure. One is best advised in these cases to either cull or isolate sick birds. The birds should be kept warm, well fed, watered and stress-free to see if the birds can recover from the disease (Anon, 2012).

2.11.2 Prevention and Control

2.11.2.1 Biosecurity: Prevention and control measures against IB mainly include strict biosecurity, good hygiene and sanitation practices, along with judicious vaccination program. All-in/all-out' operation of rearing of birds, cleaning and disinfection between batches will reduce the level of infection. Good management practices comprise of strict isolation and quarantine, restocking with disease free day old chicks (Welchman *et al*., 2002; Sylvester *et al*., 2005).

Steps to minimize the intensity of infectious virus and limiting its introduction in to poultry houses include controlling visitors' access to the farm premises; keeping separate clothing, footwear and equipment for each unit; controlling movement of farm personnel and equipment between farms and keeping appropriate footbaths with disinfectants at the entry points. These check points are very crucial for prevention and control of IBV on multi-age farms. All organic materials should be removed and disposed from the poultry house and cleaning the houses at 35-55 Bar water pressure (adding detergents in the cleaning process is also advisable) (De wit *et al*., 2010). Even though IBV is easily killed, use of suitable disinfectants (formaldehyde or chlorine releasing, quaternary ammonium compounds) with appropriate concentration and an optimum working time is very crucial to minimize infectivity of any residual

virus particles. Disinfectants can be very helpful for preventing virus infections in a farm. Successive chicken flocks must be restocked with a minimum of 10-14 days downtime between them (De wit *et al*., 2010; Dhama *et al*., 2011a).

2.11.2.2. Vaccination: Vaccination is the main method for the prevention and control of infectious bronchitis virus. In most of the countries of the world, day-old chicks are being vaccinated in hatcheries with low virulent IBV vaccines. Thereafter, a booster immunization is followed with virulent vaccines, usually in drinking water. This low virulence is suitable for chicks with a lower level of maternal immunity and they do not cause respiratory reactions which can occur with vaccines of higher virulence; disadvantage being that the low level of immunity can only protect the respiratory tract (Kataria *et al*., 2005). There are two types of vaccine, live attenuated and inactivated killed oil emulsion vaccines. Live vaccines are used in broilers and for the initial vaccination of breeders and layers while inactivated (oil emulsified) vaccines are administered in breeders and layers mainly during laying (Ladman *et al*., 2002; Jackwood *et al*., 2009). Nevertheless, for inactivated vaccines to be effective birds should have been previously "primed" with a live vaccine. Serial passages of IBV strains are followed in embryonated chicken eggs (ECE) in order to achieve the required level of attenuation so as to be used in live vaccines. With optimal conditions, vaccination may give immunity for many months and this may be life-long (Cook, 2001; Bijlenga *et al*., 2004).

a. Live Vaccines

These are Massachusetts strains: H120 vaccine (the most common representative of live vaccines) which is a mild vaccine and is known for the typical level of attenuation due to the number of passages it has undergone. It is usually used

for primary vaccinations without inducing a long lasting immunity, in areas where there is increased level of field challenge with the intention of keeping the local protection of the respiratory tract at high level. Initial vaccination can be done individually by eye drop, intra tracheal or intranasal route or by mass vaccination (e.g., coarse spray or drinking water). Such procedure is usually inexpensive; induce local as well as systemic immunity. But unfortunately it can cause some vaccination reaction observed for a few days after vaccine application (Matthijs *et al*., 2003; Bijlenga *et al*., 2004). Ma5, a single component vaccine, is a mild one, used as a single component and can also be included in first vaccination programs with IB 4/91 vaccines and inactivated vaccines for broad protection against different IBV serotypes. Live vaccines are generally used in breeders and layers (young birds) to keep a good level of local protection of the respiratory tract and are advisable in areas of increased level of field challenge. However, selection of vaccine strain must be based on the strains prevalent in the area/country. Different vaccine strains in Specific Pathogen Free Chicken (SPF) provides cross-protection against homologous and reference strains and variant field isolates (De Wit and van de Sande, 2009). Higher levels of cross-protection to some heterologous strains are given by combination of Mass and Conn or Mass and JMK. The occurrence of multiple serotypes of the virus has complicated and increased the cost of disease prevention and warrants the use of local strains in vaccines for their effective control. The inactivated vaccines are used primarily at the point of lay to avoid stress and loss of production. The Massachusetts (Mass or M41) strain is the most popular one as it is the representative of the initial isolates reported from many countries (Gelb *et al*., 1991, 2005; Cook *et al*., 1999; Terregino *et al*., 2008). In order to give specific protection against the IBV type, IB 4/91 variant virus (containing a strain of the 4/91serotype) or IB 274 vaccine virus

(containing a strain of the D207(D274) serotype) are used; when combined with Ma5 and IB multi vaccines, they provide broad protection (Mase *et al*., 2008).

b. Inactivated Vaccines

Inactivated vaccines induce long lasting immunity, show no vaccination reactions, usually cost more than live vaccines and combination of different antigens apart from IBV can be achieved when given individually. Elevated levels of circulating antibodies were stimulated by inactivated vaccines than live vaccines; therefore they are useful in a breeder program where maternal antibody protection is needed (Ladman *et al*., 2002). Still, due to induction of better T cell responses and rendering a higher local antibody (IgA) stimulation, modified live vaccines play significant role in protecting commercial layers (Ladman *et al*., 2002). Chickens must be properly primed with live vaccines, in order to exploit the potential of the inactivated vaccine and by this way, highest titres will be obtained at an interval of at least 4-6 weeks (period of vaccination between last live and inactivated vaccine) (Ladman *et al*., 2002). Further, vaccination programs may be simplified by combining inactivated antigens against two or more serotypes (or against two or more diseases) into one vaccine (Hong *et al*., 2012).

c. IB Vaccines Employed in Various Countries

Both modified live vaccines and inactivated water-in-oil emulsions are available for the Massachusetts, Connecticut and Arkansas serotypes in North America; California strains and Georgia 98 vaccines being used in USA. In Europe, "Holland variants" commonly designated by number (e.g., D-274, D-1466) are documented; IB H120 based vaccine being used in most parts of Europe. IB Ma5 and IB 4/91 (as mentioned earlier) are live, freeze-dried vaccine serotypes, giving long

lasting protection. Infectious Bronchitis D274 is a live vaccine against strain D274 in poultry for vaccination of future breeding and layers stock. Vic S vaccine is used in most vaccination programs in Australia. The K2 vaccine might be useful for the control of newly evolving IBV recombinants (new cluster 1) and variants (new cluster 2) in Korea (Lim *et al*., 2012). H120 and M41 are the widely used IBV vaccines in Nigeria (Ducatez *et al.,* 2009)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Sokoto State which is geographically located in the North western part of Nigeria between the longitudes $4^0$8'E and $6^0$54'E and latitudes 12^0N and $13^0$58'N. The state shares borders with Niger republic to the north, Kebbi State to the south and Zamfara State to the east (NPC, 2006). It is located in the extreme northwest of Nigeria, near to the confluence of the Sokoto River and Rima River. Sokoto State covers a total land area of about 32,000 square kilometers with an estimated human population of 4,244,399 and poultry population of 3,869,355 (NPC,

2006). The state is in the dry Sahel and has 23 local governments for administrative purposes and farming is the major occupation of majority of its inhabitants. Rainfall is between June and September in the northern part of the state, and April to October in the southern part. The average annual rainfall is 600mm with most of it falling in July and August (Mohammed and Baba, 2013). The state is divided into four agricultural zones made up of 4-7 LGAs each, namely Isa (Isa, Goronyo, Sabon Birni, Wurno and Rabah); Gwadabawa (Gwadabawa, Illela, Gada, Binji, Tangaza, Gudu and Silame); Tambuwal (Tambuwal, Kebbe, Yabo, Shagari) and Sokoto (Sokoto north, Sokoto south, Wamakko, Kware, Dange, Bodinga and Tureta) (MAHFD, 2015). The state is characterized by two extreme temperatures relative to its tropical position, the hot and the cold seasons with highest temperatures experienced in the months of March/April (45^0C day time temperature) and lowest temperatures during harmattan between November and February (about 28.3^0C day time temperature) (Collin, 2005).

3.2 Study Design

The research was designed to be a prospective cross sectional study. The work was divided into three studies viz:

I. Serology
II. PCR
III. Sequencing and Phylogenetic analysis

3.3 Sample Size Determination

The minimum sample size for the work was determined using the formula:

$N = Z^2.P.(1-P)/d^2$ (Thrushfield, 2007)

Where

N = sample size, Z =1.96, as standard error at 95% confidence interval, P = previous prevalence and d = level of precision (5%). For this study, P was estimated to be 89% based on prevalence reported by Mungadi *et al.* (2015).

$N = (1.96^2) \times 0.89 (1-0.89)/ (0.05)^2$

N = 3.8416x0.89 x 0.11/0.0025

N = 0.376/0.0025 = 150

3.4 Sampling Frame

A total of 400 chickens (indigenous/local and exotic/commercial) were sampled for serology. Tracheal and cloacal swabs from 150 chickens manifesting clinical signs of IB were taken for RT-PCR, making 300 samples (150 tracheal swabs and 150 cloacal swabs). These comprised of indigenous (local) and exotic (commercial) chickens from each of the four agricultural zones of the state. Local chickens were sampled from live bird markets and back yard poultry, while commercial chickens comprising of broilers, pullets and layers were sampled from commercial poultry houses and backyard poultry flocks situated across the state. Types of chickens, age and sex were used as sampling frame in this work. Tables 3.1 and 3.2 show the sampling frame and the number of different variables was taken considering availability and owners' cooperation.

3.5 Sampling Method

Purposive sampling method was adopted in this study as described by Paul (2006). It is a type of non-probability sampling where individuals with similar characteristics are selected. The method was used for sampling in terms of location (areas with poultry markets and poultry farms) and for taking tracheal and cloacal swabs for PCR (chickens manifesting clinical signs of IB).

Simple random sampling method was also adopted as described by Valerie and John (1997). It is a type of sampling where a group of subjects (a sample) is selected for study from a larger group (a population). Each individual is chosen entirely by chance and each member of the population has an equal chance of being selected. This method was applied for choice of chickens in selected areas.

Local (indigenous) chickens were sampled from live bird markets and households while commercial (exotic) chickens were sampled from commercial poultry farms and households in the four agricultural zones of the state viz; Achida, in Isa zone, Illela in Gwadabawa zone, Tambuwal in Tambuwal zone and Sokoto in Sokoto zone. Chickens less than six months of age were considered as young while those above six months were considered as adults.

3.6 Sample Collection and Preservation

Samples were taken between November 2016 and February 2017. Blood samples were taken from chickens in poultry markets; households' poultry and commercial poultry houses from selected areas across the state, sera were harvested and tested for the presence of infectious bronchitis virus antibodies using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit source from Katja Noor - Corouge with product code IBVS-5P (ID Screen France). From each chicken, 1-2 mLs of blood was collected through the brachial (wing) vein aseptically as described by Kelly (2013). Blood collected were placed in labeled sterile plain serum bottles and sera were harvested by centrifuging at 4,000 rpm for t minutes. Harvested sera were pipetted and stored in cryovials at -20^0C in the Central Research Laboratory Faculty of Veterinary Medicine City Cmapus UDUS until use.

Tracheal and cloacal swabs were as well collected for viral RNA isolation and characterization using Reverse-Transcription Polymerase Chain Reaction (RT-PCR) technique. Each swab was placed in a sterile tube containing 1mL of Phosphate buffered saline (PBS) as described by Martin *et al.*, (2006) and transported to laboratory in cold conditions. The swabs were then scrapped on the side of the tube to facilitate removal of contents from the swab head. Swabs from three route were pooled together (100 pooled tracheal and cloacal swabs\) and stored at -20^{0}C until analysis as described by Liu (2009) and Jonathan (2016).

Table 3.1: Sampling frame for Serology

Variables	**Number**
Exotic (commercial)	205
Indigenous (local) 0020	195
Adults	219
Young	181
Male (cock)	183
Female (hen)	217
Sokoto	250
Tambuwal	50
Gwadabawa	50

Isa	50

Table 3.2: Sampling frame for RT-PCR

Variables	**Number**
Exotic	32
Indigenous	52
Adults	34
Young	50
Male	34
Female	50
Sokoto	50
Tambuwal	6
Gwadabawa	12
Isa	16

Table 3.3: Areas where samples were collected

Local Chickens	**Commercial Chickens**
Sokoto meat and vegetables market	Tabanki farms Sokoto
Unguwar Rogo poultry market	Maska farms Sokoto
Achida Market	Dan Uda Farms Sokoto
Illela Market	Ambarura farms Guiwa Lowcost Sokoto
Tambuwal market	A backyard Poultry Mabera Sokoto

Two households in Achida	Two backyard Poultry in Lowcost Sokoto
Two households in Illela	Three backyard Poultry in Tamaje Sokoto
Two households in Tambuwal	Three backyard poultry in Achida
	Three backyard poultry in Illela
	Three backyard poultry in Tambuwal

3.7 Study I Serology

ID Screen Infectious Bronchitis ELISA kit sourced from Katja Noor - Corouge with product code IBVS-5P (ID Screen France) was obtained and stored at -20^{0}C according to the manufacturer's recommendations. The kit was used for serological survey of antibodies against IBV from serum samples collected. It is a quantitative test for the detection of IBV-specific antibodies in chicken sera; the result was read at wave length of 450nm. The procedure was carried out in the Central Laboratory City campus, Usmanu Danfodiyo University, Sokoto. All samples and reagents were

allowed to attain room temperature before assay. The test principle for ELISA as described by the manufacturer is in appendix A.

3.8 Study II Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.8.1 Viral RNA Extraction

Viral RNA extraction Mini kits QIAamp® (QIAGEN® Sample and Assay Technologies products sourced from Germany) with product code 52904 were used for RNA extraction as described by the manufacturer. (QIAamp®) Viral RNA mini Kits represent a well established general-purpose technology for viral RNA preparation. The kits combine selective binding properties of a silica-gel-based membrane with the speed of microspin or vacuum technology and they are ideally suited for simultaneous processing of multiple samples. They were stored at room temperature before use. The procedure was carried out in the Central Laboratory City campus, Usmanu Danfodiyo University, Sokoto.

The samples were first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions were then adjusted to provide optimum binding of the RNA to the QIAamp® membranes, and the samples were loaded onto the QIAamp® mini spin columns. The RNA were expected to have bound to the membranes and contaminants were efficiently washed away in two steps using two different wash buffers. High quality RNA was eluted in a special RNase free buffer ready for storage. The purified RNA were free of protein, nucleases, and other contaminants and inhibitors.

The special QIAamp® membrane guarantees extremely high recovery of pure, intact RNA in just 20 minutes without the use of phenol/chloroform extraction or alcohol precipitation. All buffers and reagents were guaranteed to be RNase-free.

The protocol for RNA extraction is in Appendix B.

Purity and concentration of the 100 extracted viral RNA were calculated by determining the ratio of the absorbance at 260nm to the absorbance at 280nm using a J.P Selecta series 2005® UV spectrophotometer. The procedure was carried out in Central Research Laboratory, Faculty of Veterinary Medicine City campus UDUS.

3.8.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

IBV vaccine-strain H120 provided by Dr. Faruku Bande Universiti Putra Malaysia and Sokoto Zonal Veterinary Clinic was used as positive control to optimize the RT-PCR program.

i. Reverse transcription (cDNA synthesis)

The extracted RNAs were reverse transcribed to form cDNA. This was performed with **5**µL of denatured RNA sample, 2µL of gDNA remover and 7uL of RNAse free water. The mixture was incubated at 42^0C for 2 minutes. This was then added to another mixture containing 1µL of reverse primer, 4µL of quantiscript® RT-buffer and 1µL of quantiscript® reverse transcriptase and incubated at 42^0C for 30 minutes and inactivated at 95^0C for 3 minutes. The cDNA was stored at -20^0C.

ii. Polymerase Chain Reaction (PCR)

Integrated DNA Technologies IDT® primers (Coral vial, IOWA, USA) were used. The cDNA was screened for IBV using PCR targeted to the highly conserved 3'UTR of the virus genome with primers designed by Cavanagh *et al.* (2002) and provided by Dr. Faruku Bande. Forward **(**Sense specific) primer UTR11 was first used in combination with reverse (anti-sense specific) primer UTR 41 to amplify a 266 base pair DNA fragment (Table 3.3).

For this round of PCR, 3µL of cDNA sample was used with 12.5µL QIAGEN® master mix (MgCl2, dNTP) 2X (Coral vial, IOWA, USA) 2.5µL each of forward and reverse primers and 4.5µl of RNAse-free water to a final volume of 25µL. The mixture was transferred into AB® thermocycler (Applied biosystem). The thermal profile for the PCR was 94 ˚C for 3 minutes, followed by 25 cycles of 94 ˚C for 30 seconds, 48 ˚C for 30 seconds and 72˚C for 1 minute, followed by 72^0 C for 10 minutes.

The products were screened for the presence of a specific fragment in comparison with a GeneRuler 100 bp DNA Ladder (Thermo specific) after electrophoresis was done using 1.5% agarose gel and stained with ethidium bromide DNA Gel stain (Sigma Aldrich® Missouri USA) and 1X TBE buffer according to standard (85V, 3.00A,300W) for 45 minutes. Positive bands were checked on the gel under UV-light using BioRad® gel imager.

Samples positive to the screening PCR were subjected to full amplification of *S1* coding region. IBV vaccine-strain H120 was used as positive control and molecular grade water as negative control. A pair of primers for amplifying the entire *S1* gene (GBR1 and GBR2) designed by Fang *et al*. (2011) was used (Integrated DNA Technologies IDT® primers). Sequences of the forward primer (GBR1) and reverse primer (GBR2) are shown in table 3.3. The anticipated amplicon was about 1800bp, encompassing the entire *S1* region.

The PCR reaction (25 uL) contained 3uL of cDNA (about 100 ng), 12.5uL of 10x PCR master mix (qiagen) 2 uL of 10 mMol of each of the two primers (forward and reverse),7.5 uL of RNAse- free water. The PCR conditions for amplification were 94^0 C for 5 minutes, 30 cycles of 94^0 C for 40 seconds, 60^0 C for 40 seconds, and 72^0 C for 2 minutes, followed by 72^0 C for 10 minutes.

Electrophoresis using agarose gel was done as in the first round of PCR and viewed under UV light for positive bands. Amplicons of the positive samples were sent to Inqaba Biotechnical Industries West Africa, Ibadan which were further transported to the Industry's main branch in Pretoria, South Africa for sequencing.

3.9 Study III Sequencing and Phylogenetic Analysis

3.9.1 Sequencing

PCR products were purified using a Monarch® PCR & DNA clean up kit (Biolabs®) following the instructions of the manufacturer. DNA (10–100 ng) was sequenced in both directions with a Big Dye™ Terminator version 3.1 cycle sequencing kit (Applied Biosystems™) Sequence studio genetic analyzer; (Applied Biosystems) using the PCR primers (IDT®) as sequencing primers.

Table 3.4: Primers used in the study

Name	Sequence (5′ to 3′)	Target Region
UTR11	GCTCTAACTCTATACTAGCCTA	UTR
UTR41	ATGTCTATCGCCAGGGAAATGTC	UTR
GBR1	ATGTTGGTGAAGTCACTGTTTATA	*S1* gene
GBR2	ATACGCGTTTGTATGTACTCATCTG	*S1* gene

UTR: Cavanagh *et al*. (2002), GBR: Fang *et al*. (2011)

3.9.2 Data Analysis

Chi Square and binary logistic regression were used to analyze the results of serology. Basic Local Alignment Search Tool (BLAST) was used to determine sequence identities and query cover. The obtained sense and anti-sense sequences were submitted for quality evaluation using Phil's Read Editor (Phred) online application (Togawa and Brigido, 2012). The sequences were assembled together with the Cap-Conting application in Bioedit 7.0.9.0 software. Multiple Sequence Comparison with Log Expectation (MUSCLE) was used to align the sequences with the reference sequences downloaded from National Center for Biotechnology Information (NCBI) database as recommended by Chenna *et al*. (2003) using MEGA 7 software (Kumar *et al*., 2016). The software was used to construct a nucleotide Phylogenetic tree (Neighbor- joining, 1,000 bootstrap replications). IBV sequences from all relevant reference strains available on GenBank (NCBI) were used for comparison. The tree was used to determine the genotype of the sequenced found IBV strains. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and Nei, 2004). The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 80 positions in the final dataset.

CHAPTER FOUR

RESULTS

4.1 Over All Prevalence

Out of the 400 sera tested using indirect ELISA, 346 were positive (86.50 %) and out of 84 samples tested for PCR, 11 were positive (13.09%). The distribution is shown in table 4.1.

4.2 Seroprevalence

The seroprevalence of infectious bronchitis virus in indigenous (local) chickens showed out of 99 adult male chickens sampled, 87.88% tested positive, and out of 48 adult females, all (100%) were positive, out of the 24 young males 95.83% were positive and out of 24 young females 62.50% were positive (Table 4.2). The seroprevalence of infectious bronchitis virus in exotic (commercial) chickens sampled showed that, out of 85 broiler chickens tested, 76.47% were positive, out of 72 screened layers, 84.72% were positive and out of the 48 screened, pullets 97.92% were positive (Table 4.3).

Table 4.1: Summary of ELISA and PCR Assay

Test	**Total number**	**Positive (%)**	**Negative (%)**
ELISA	400	346(86.50)	54(13.50)
RT-PCR	84	11(13.09)	73(86.10)

Table 4.2: Seroprevalence of Infectious Bronchitis Virus in Indigenous (Local) Chickens in Sokoto State

Local birds	**Positive (%)**	**Negative (%)**	**Total**
Adult male	87 (87.88)	12 (12.12)	99
Adult female	48 (100.00)	0 (0.00)	48
Young male	23 (95.83)	1 (4.17)	24
Young female	15 (62.50)	9(37.50)	24
Total	173 (88.72)	22(11.28)	**195**

Table 4.3: Seroprevalence of Infectious Bronchitis Virus in Exotic (Commercial) Chickens in Sokoto State

Commercial (exotic) birds	**Positive (%)**	**Negative (%)**	**Total**
Broilers	65 (76.47)	20 (16.95)	85
Layers	61 (84.72)	11 (15.28)	72
Pullets	47 (97.92)	1 (2.08)	48

Total	173 (84.39)	32(15.61)	**205**

The seroprevalence of infectious bronchitis virus in local and commercial (exotic) chickens showed that out of 195 local chickens tested, 88.72% were positive, and out of 205 commercial chickens tested, (84.39%) were positive. (Table 4.4)

The seroprevalence of Infectious bronchitis virus in young and adult chickens sampled showed that, out of the tested 219 adult chickens, 89.50% were positive and out of 181 young, 82.87% were positive. (Table 4.5)

The prevalence of infectious bronchitis viral antibodies in cocks and hens chickens sampled showed that, out of 183 cocks tested, 86.88% were positive and out of 217 hens tested, 86.17% were positive. (Table 4.6)

4.3 PCR Result

From the 100 extracted viral RNA, 84 passed purity and concentration test (Appendix C). The 84 samples were tested and 11 (13.1%) were positive for infectious bronchitis virus after the first round of PCR using UTR primers. They are as follows:

1. TR/B/SK/05
2. TR/B/SK/06
3. TR/B/SK/23
4. TR/AF/SK/07
5. CL/AM/SK/06
6. TR/YM/IS/15
7. CL/YM/IS/15
8. CL/P/SK/05
9. CL/YM/GW/08

10. TR/AF/IS/04

11. TR/YF/SK/16

The Samples were designated as route/type/location/sample ID number. TR=Trachea; CL=Cloaca; B=Broiler; P= pullet; AF=Adult female (Local); AM=Adult male (Local); YM=Young male (Local); YF=Young female (Local); SK=Sokoto; GW=Gwadabawa; and IS=Isa.

From the results, there were 7 positives from tracheal swabs and 4 positives from cloacal swabs. The PCR results showed that out of the 11 positive samples obtained, 4(36.36%) were from exotic chickens while 7 were from indigenous ones (63.64%). Out of the 84 tested, 4.76% of the exotic were positive and 8.33% of the local chickens were positives. Out of all exotics tested, 12.5% were positive and out of all local ones tested, 13.5% were positive. There were three positive adults (27.27%) and eight positives from young chickens (72.73%). Out of the total number (84) tested, adults had 3.57% positives and young chickens had 9.53% positives. Out of the adults tested, 8.83% were positive and out of the young ones tested, 16% were positive. Out of the 11 that were positive, six (54.55%) were cocks and five (45.45%) were hens. Out of the 84 tested, cocks had 7.15% positives and female chickens had 5.95% positives. Out of all the cocks tested, 17.65% were positive and out of all the hens tested, 10% were positive.

Table 4.4: Seroprevalence of Infectious Bronchitis Virus in Indigenous (Local) and Exotic (Commercial) Chickens in Sokoto State

Type of bird	**Positive (%)**	**Negative (%)**	**Total**
Local (indeginous)	I73 (88.72)	22 (11.28)	195
Commercial (exotic)	173 (84.39)	32 (15.61)	205
Total	346(86.50)	54(13.50)	**400**

There was no statistical significance with Chi-value 1.603, CI (confidence interval) 95%, P value = 0.205.

Table 4.5: Seroprevalence of Infectious Bronchitis Virus in the Two Age Groups of Chicken in Sokoto State

Age	Positive (%)	Negative (%)	Total
Adult	196 (89.50)	23 (10.50)	219
Young	150 (82.87)	31 (17.13)	181
Total	346 (86.50)	54 (13.50)	400

There was statistical significance with Chi-value 3.179, CI (confidence interval) 95%, P value = 0.038.

Binary logistic regression test was carried out and there was no significance with P= 0.093.

Table 4.6: Seroprevalence of Infectious Bronchitis Virus in the Two Sexes of Chickens in Sokoto State

Sex	Positive (%)	Negative (%)	Total
Males	159 (86.88)	24 (13.12)	183
Females	187 (86.17)	30 (13.38)	217
Total	346 (86.50)	54 (13.50)	400

There was no statistical significance with Chi-value 0.043, CI (confidence interval) 95%, P value = 0.836

From chickens tested in Sokoto zone for PCR, seven (63.64%) were positive, no positive was obtained from those tested in Tambuwal zone (0%), Gwadabawa zone had one positive (9.09%) and Isa zone had three positives (27.27%). Out of the 84 tested, Sokoto zone had 8.34% positives, Tambuwal zone had 0% positive, Gwadabawa had 2.38% positives and Isa zone had 7.14% positives. Out of all the chickens that were tested from Sokoto zone, 14% were positive, 0% was positive

from Tambuwal zone, out of those tested from Gwadabawa zone 8.33% were positive and out of all those tested from Isa zone, 18.75% were positive (table 4.7).

From the gel image in the first round of PCR where 266bp was amplified using UTR primers and Biolabs® 100bp ladder, there was appearance of bands corresponding to the targeted position on DNA ladder, indicating the amplicons that were positive (figure. 4.1).

Table 4.7: Results of the PCR in Chickens of various Categories

Variables	Number tested in the category and percentage positive (%)	(%)Positives out of total number (84) tested	Number positive out of all positives (%)
Exotic	32 (12.50)	4.76	4 (36.36)

Indigenous	52 (13.50)	8.33	**7** (63.64)
Adults	34 (8.83)	3.57	3 (27.27)
Young	50 (16.00)	9.53	8 (72.73)
Male	34 (17.65)	7.15	6 (54.55)
Female	50 (10.00)	5.95	5 (45.45)
Sokoto	50 (14.00)	8.34	7 (63.64)
Tambuwal	6 (0.00)	0.00	0 (0.00)
Gwadabawa	12 (8.33)	2.38	1 (9.09)
Isa	16 (18.75)	7.14	3 (27.27)

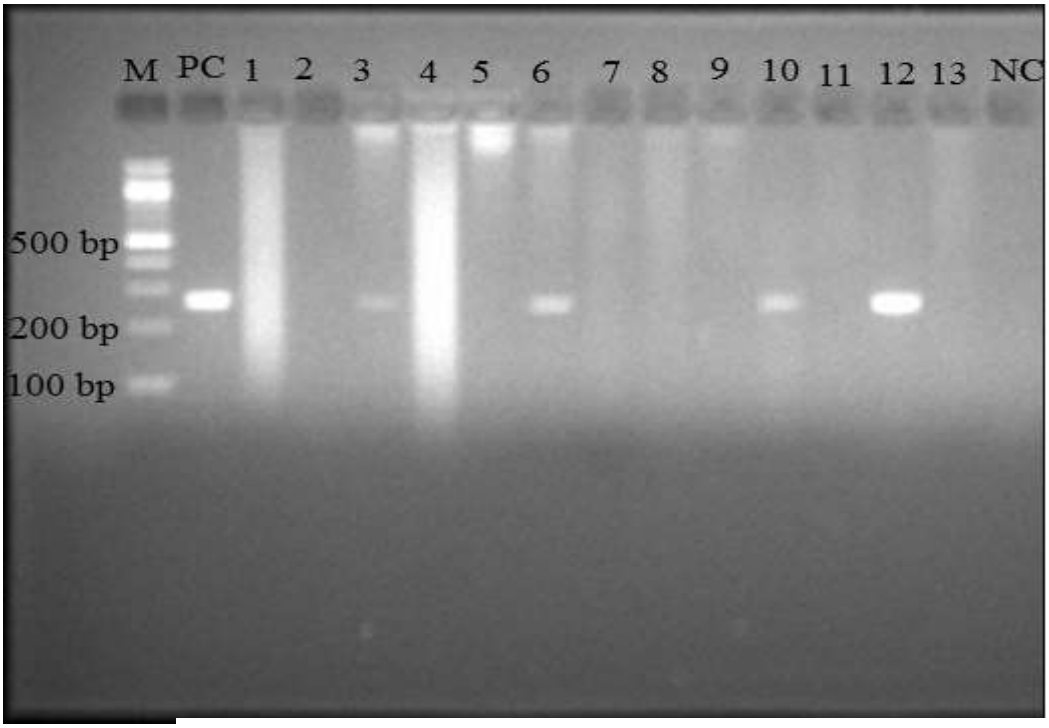


Figure 4.1: Representative of elecrophoresis gel images for the first round of PCR (using UTR primers)

The second round of PCR showed amplification of 1,800bp fragment using primers that amplify the full length *S1* coding region of the virus; there was appearance of bands corresponding to the targeted position on DNA ladder (Promega® 1Kb ladder), indicating the amplicons that were positive.

After second round of PCR (full *S1* gene amplification) and gel electrophoresis, the products were viewed using Biorad® imager as in the first PCR, all the 11 PCR products were positive (figure 4.2).

4.4 Sequencing and Phylogenetic Analysis

The hyper variable region of the S1 gene was sequenced and compared with reference IBV sequences available in GenBank (Fig. 4.1). After processing the positive samples, the hypervariable region of the *S1* gene was sequenced and 5 DNA sequences were obtained. The samples from which the sequences were obtained are:

1. TR/B/SK/05

2. TR/B/SK/O6

3. TR/AF/SK/07

4. TR/YM/IS/15

5. CL/YM/IS/15

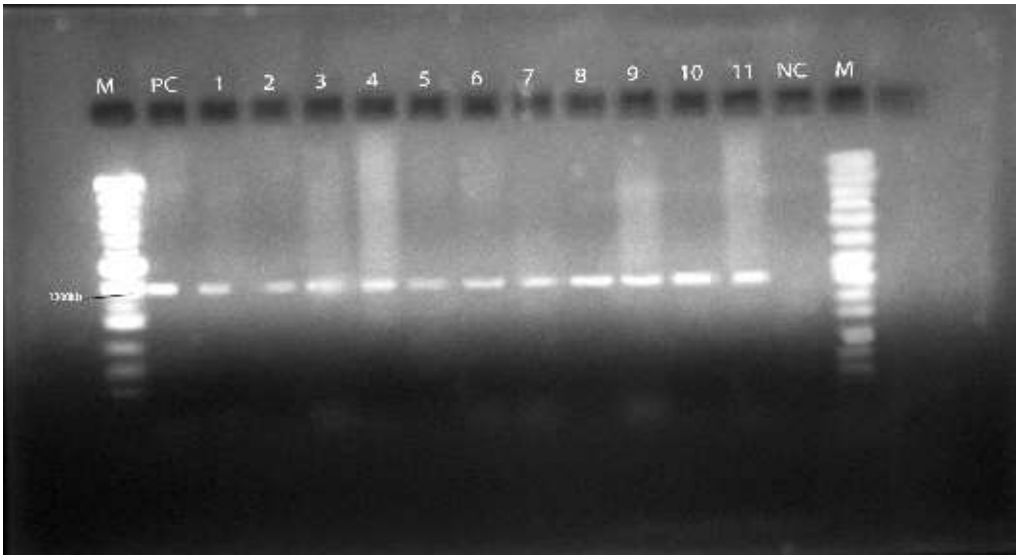


Figure 4.2: Elecrophoresis gel image of second round of PCR (using *S1* primers)

Samples 1 and 2 belonged to broiler chickens from same back yard poultry in Mabera area Sokoto. Sample number 3 was from local adult hen from Sokoto meat and vegetable market. Samples 4 and 5 were obtained from local young cocks from Achida market.

The sequencing results (figure 4.4) showed that nucleotide sequences obtained from samples TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07 and TR/YM/IS/15 (figure 4.3) are most closely related to NGA/A11637/2006 accession number FN430415 (GenBank) reported from Nigeria, while nucleotide sequences obtained from CL/YM/IS/15 is most closely related to patridge/GD/S14/2003 strain accession number AY636283 (GenBank) reported from Asia.

Following the results of sequencing, BLASTing (figures 4.3 and 4.4) and Phylogenetic analysis (Phylogenetic tree Fig.4.5) in this study, one of the IBV strains characterized was found to be closely related to the Asian strain patridge/GD/S14/2003 strain accession number AY636283 with 82% nucleotide sequence similarity 50% query cover. The strain was also found to be closely related to Chinese IBV strains gamma CoV/ck/china/1101/16 and IBV virus strain YX10 with accession numbers KY62011 and JX840411 respectively which clustered on the Phylogenetic tree both with 80% nucleotide sequence similarity. While four of the strains were closely related to 'Ibadan' strain (NGA/A116E7/2006) accession number FN430415 a variant unique to Nigeria reported by Ducatez *et al*. (2009) named according to the location where the reference virus was found with 82% nucleotide sequence similarity, 91% query cover. Alignment of sequences is shown in Appendix D.

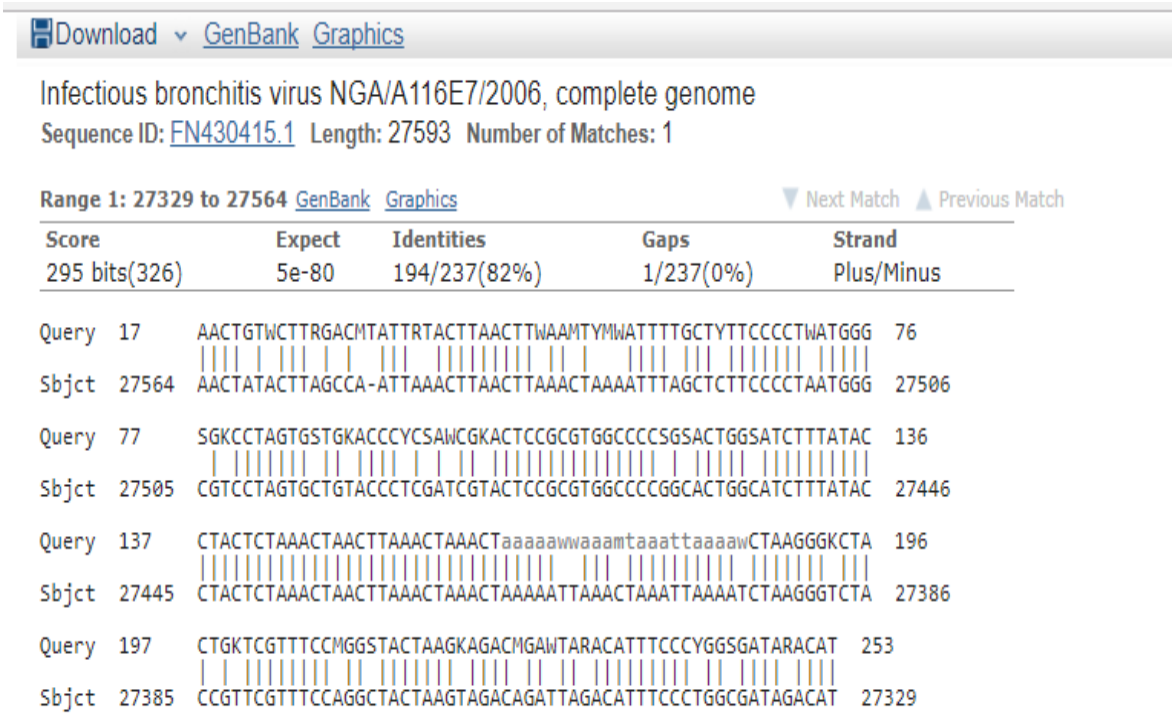


Figure 4.3: Partial Sequence Analysis Results of IB Virus Gene

Note: (i) Querry = local strain

(ii) Subject = reference Genebank sequence (NGA/A116E7/2006).

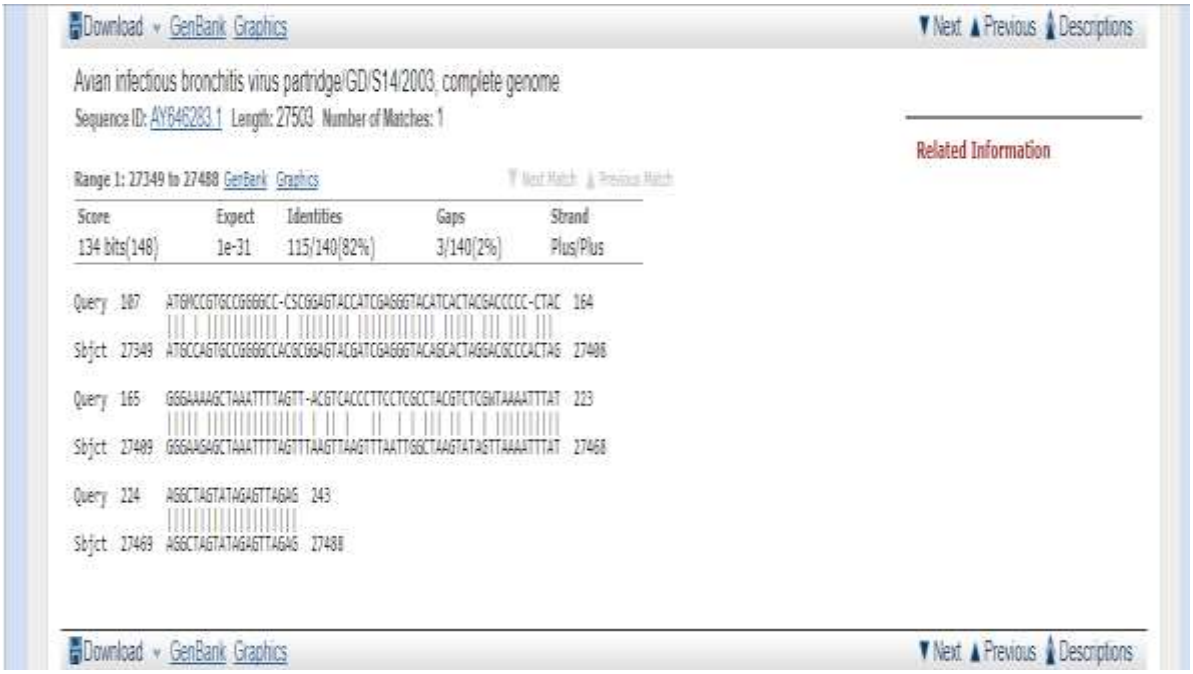


Figure 4.4: Partial Sequence Analysis Results of IB Virus Gene

Note: (i) Querry = local strain

(ii) Subject = reference Genebank sequence (patridge/GD/S14/2003)

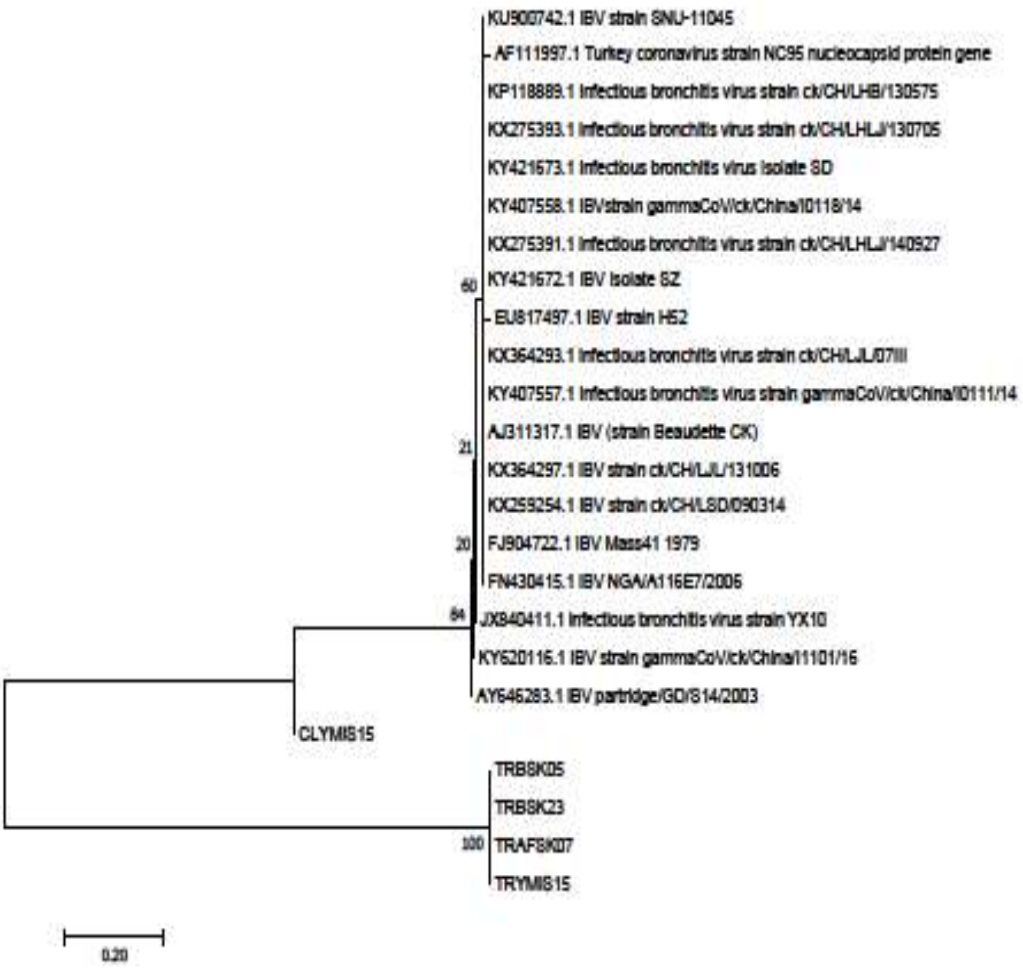


Fig.4.5: Phylogenetic Analysis of the Full-length IBV *S1* Gene Strains from Sokoto State at Nucleotide Level.

The tree was constructed with the Neighbour joining method (Maximum composite likelihood) with 1000 bootstrap replicates. TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07, TR/YM/IS/15, CL/YM/IS/15 and some IBV reference strains were included. Bar 0.20 nucleotide substitutions per site. The evolutionary history was inferred using the Neighbor-Joining method (Saito and Nei, 1987). The optimal tree with the sum of branch length = 2.07563859 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

CHAPTER FIVE

DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The overall prevalence of 86.50 % was obtained after running ELISA. It is therefore evident that the prevalence of avian infectious bronchitis in Sokoto State is still high which is close to what was obtained by Mungadi *et al*. (2015) of 88.82% in similar studies in the year 2013. This is also close to the prevalence of 84% and 82.% obtained by Owoade *et al.* (2006) and Emikpe *et al.* (2010) respectively after carrying out a serosurvey of antibodies to infectious bronchitis in chickens in southwestern Nigeria. Dergham *et al.* (2009) also reported seroprevalence of 92.9% in all flocks tested in Jordan using hemagglutination inhibition (HI) test which is higher than prevalence obtained in this work. It is important to note that the prevalence in this study is slightly lower than 90% obtained by Ducatez *et al.* (2004) who examined serum samples of 52 flocks for the presence of IBV antibodies from poultry farms in Nigeria using a commercial ELISA kit.

Prevalence of infectious bronchitis viral antibodies was found to be highest in Sokoto zone. This might not really indicate highest prevalence of the disease in that area, because chickens are transported from one market to another across the state which makes it difficult to trace their original source. Statistically, there were no significant differences between the results obtained in these zones. This may be associated with similarity in the environmental conditions throughout the state, breeds raised and systems of management.

The results showed high prevalence in both local and commercial chickens but with statistically insignificant higher percentage in local breeds, this is similar to report of Soos *et al.* (2008) who reported that sero-positivity to IBV and other common poultry diseases were relatively high among both back yard and broiler chickens tested in Galapagos Island, Ecuador. Emikpe *et al.* (2010) also obtained high seroprevalence in both indigenous and exotic breeds but Mungadi *et al*. (2015) reported insignificant difference between the two types of chicken although the prevalence was higher in the exotic chickens. The difference observed in this study may be due to strict biosecurity measures imposed in commercial poultry farms which are not usually instituted in local chicken rearing.

The higher seroprevalence in laying type of chickens seen in this work supports the findings of Koenen *et al.* (2002) on immunological differences between layer- and broiler-type chickens. The results suggested that broilers are specialized in the production of a strong short-term humoral response and layer-type chickens in a long-term humoral response in combination with a strong cellular response, which is in conformity with their life expectancy. In this work, seroprevalence of 84.7% was reported in layers which is lower than the prevalence reported by Ahmed *et al*. (2007) who reported 100% sero-positivity to IBV in number of layer flocks tested in Pakistan and 66.6% in number of broiler flocks.

Higher seroprevalence was obtained in adults than in young which was statistically insignificant. This is in line with the work of Mungadi *et al*. (2015) where adults had higher prevalence that was statistically significant. The higher prevalence in adults in this study even though not significant statistically, could be attributed to the fact that adults are being more exposed to risks of infections due to transportation

from one market to another. The finding also supports the work of Ducatez *et al.* (2004) where there was no serological significant difference in respect with age in commercial (exotic) chickens tested for IBV in western Nigeria.

The sero-positivity to IBV was found to be high and almost equal in the two sexes. This does not support the findings of Paul *et al.* (2008) where higher prevalence of 58.33% was obtained in female chickens compared to low prevalence of 25% in males tested using ELISA in Bangladesh. But there was statistically insignificant higher percentage in males reported by Mungadi *et al*. (2015).

This is the first work in which tracheal swabs were used for investigating IBV using RT-PCR technique in Sokoto State. The overall result of RT-PCR for full amplification of *S1* gene from tracheal and cloacal swabs from 150 sampled chickens in Sokoto State showed 13.1% positive and 86.9% negatives. This is close to 18% reported by Ducatez *et al*. (2009) in similar study conducted between 2002 and 2007 in Nigeria and Niger using cloacal swabs and lung samples. This indicates that the high sero-prevalence did not mean high level of infection with IBV. Ducatez *et al*. (2009) similarly reported that in northern Nigerian live bird markets and in Niger backyard poultry, IBV infections seemed to be less common. The detection of IBV from non-vaccinated birds by RT-PCR indicates that these flocks had experienced field exposure to IBV. The percentage positives (13.1%) recorded in this work is less than the 58.8% PCR positives reported by Dergham *et al.* (2009) in chickens tested in Jordan as well as the 63.4% in Egypt reported by Selim *et al*. (2013) after testing 205 tracheal swabs from commercial chickens using PCR in 2012.

Other researches reported high percentage than the percentage positives (13.1%) recorded in this case, 36.6% and 40% PCR positives were reported in Iran and Brazil respectively (Mohammed *et al.,* 2013; Huijbum *et al.,* 2016).

The prevalence of IBV infection in the four agricultural zones tested in this study by RT-PCR indicated widespread distribution of infectious bronchitis virus in most parts of Sokoto State except for Tambuwal zone where no positive RT-PCR result was obtained. The percentage positive in Sokoto zone showed highest prevalence in the zones (63.64%), Isa and Gwadabawa zones had lower prevalences of 27.27% and 9.1% respectively. The highest prevalence obtained in Sokoto zone might be associated with more poultry commercial activities in the zone resulting to chickens having more contact with each other when moved from one place to another which further results to contracting various diseases.

The PCR result showed higher positives in local breeds than in commercial (exotic) breeds tested (63.64%), as it was in the result of serology even though the exotic breeds also too exhibited high sero-positivity. All the exotic chickens that appeared to be PCR positive belonged to the young category of age group; these included 3 broilers and 1 pullet. This is similar to the reports of Selim *et al.* (2013) who reported higher PCR percentage positives in broilers tested (65.4%) than in layers (50%) in Egypt in another work by Dergham *et al.*, (2009) where they reported that broilers were 64% positive while 54.4% layers were positive using PCR in Jordan. It is a common knowledge that biosecurity measures are more imposed in commercial egg producing farms than in the broiler farms.

The PCR positives in the young chickens tested in this study (72.73%) are higher than in the adults higher than in the adults with 27.27% which is not in

agreement with the result of serology due to the fact that the young chickens did not have a well developed immunity compared with adult ones. Male chickens tested had 54.55% PCR positives, which is not different from the female chickens having 45.45% positives. The result is in agreement with what was obtained in serology. In this study more positives were obtained from tracheal swabs (63.64%) than from cloacal swabs (36.36%) which support the suggestion by Bande *et al*. (2017) that the upper respiratory tract is the primary replication site for IBV and initial infection starts at the epithelium of Harderian gland down to lower respiratory tract before reaching urogenital and gastro intestinal tracts.

The sequenced strains in this study were not found to be related to vaccine strains but Ducatez *et al*. (2009) reported 3 strain that clustered with vaccine strains and wild type strain D274 which they attributed to recombination between Mass like and D274 strains. The TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07 and TR/YM/IS/15 strains that clustered together in this study might have evolved as a result of mutation of NGA/A116E7/2006 (Ibadan) strain considering the 82% similarity with transportation of chickens especially chicks from South western part of the country to North Western and North Eastern parts of Nigeria further increase chances of finding the presence of this strain in Sokoto State. In addition, trade in local poultry from the North to South also also increases the chances of finding the Ibadan strain in Sokoto. The Ibadan like strains in this study could belong to respiratory pathotypes considering that they were obtained from tracheal swabs of chickens manifesting respiratory clinical signs of IB even though there was absence of obvious clinical signs in chickens infected with Ibadan strains as reported by Ducatez *et al*., (2009).

Obtaining a strain which was found to be closely related to patridge/GD/S14/2003 from cloacal swab of young male indigenous chicken in Isa zone (CL/YM/IS/15) and also closely related to Chinese CoV/ck/china/1101/16 and IBV-YX10 virus strains might be through migratory birds as birds that are in contact with or reared close to chickens infected with IBV could serve as carriers. There is no particular clinical sign previously attributed to patridge/GD/S14/2003.

The results obtained in this study clearly showed that the characterised IBV strains have no relationship with the strains H120 and M41some times used for vaccination. This finding supports the report of Ducatez *et al.* (2009) that the use of H120 and M41 vaccines may not protect chickens against the variant strains of IBV circulating in the environment.

The result of this study also shows dynamic changes of IBV in the sense that there are differences in nucleotide sequences between the strains sequenced in this study and the previously reported strains deposited in GenBank that were used for comparison. This complicates the establishment of vaccine strategies to control the disease.

5.2 Conclusions

From this work it was concluded that:

The results obtained from this research demonstrated high seroprevalence to IBV in the chickens sampled and the PCR result showed low prevalence of the virus. The high seroprevalence obtained in this research does not mean high level of infection at the time of sample collection. The sequenced isolates, among the circulating IBV strains in chickens in Sokoto State, are variants from the

patridge/GD/S14/2003, NGA/A11637/2006 (Ibadan) strain CoV/ck/china/1101/16 and IBV-YX10 virus strains**.** This is the first study where Chinese like recombinant strains patridge/GD/S14/2003, CoV/ck/china/1101/16 and IBV-YX10 are reported in Nigeria. The strains are not related to the strains used for vaccination in Nigera.

5.3 Recommendations

From this work it was recommended that:

1. The use of antigen detection ELISA kits is recommended for serological survey of IBV in future researches in order to obtain true picture of the infection, rather than antibody detection ELISA kits.
2. There is need to know the pathogenicity of the circulating IBV strains and test the efficacy of current vaccines against them.
3. Clinicians should always investigate IB in cases of sick chickens presented with clinical signs of the diseases.
4. Proper disinfection of veterinary clinics/hospitals and poultry farms should be ensured to minimize spread of IBV.
5. Strategies need to be adapted continuously to the field situation in Sokoto State. Indigenous (local) poultry keepers should be enlightened and encouraged by veterinarians to consider vaccination as a mean of prevention of poultry diseases like infectious bronchitis.
6. Improvement in shelter and management in local chicken production should be encouraged for easy prevention and control of diseases.
7. There is need to impose strict biosecurity measures on both local and commercial chickens to prevent contracting poultry diseases through migratory birds.

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APPENDICES

Appendix A: ELISA Protocol

Materials

i. Serum samples

ii. Multi-channel micropipettors capable of delivering volumes of 5µl, 10µl, 100µl and 200µl

iii. Disposable tips

iv. 96 well micro plate reader (Optic Ivymen® system 2100-C)

v. Distilled water

vi. Kit components :

a. Microplates coated with purified IBV antigen

b. Positive control

c. Negative control

d. Concentrated conjugate (10X)

e. Dilution buffer 3

f. Wash concentrate (20X)

g. Substrate solution

h. Stop solution

Method

The test principle: Microwells are coated with purified IBV antigen. Samples to be tested and controls are added to the wells. Anti-IBV antibodies, if present, form an antigen antibody complex. After washing, an anti-chicken horseradish peroxidase (HRP) conjugate is added to the wells. It fixes to the antibodies, forming an antigen-antibody conjugate-HRP complex.

After elimination of excess conjugate by washing, the substrate solution tetramethylbenzidine (TMB) is added. The resulting coloration depends on the quantity of specific antibodies present in the specimen to be tested:

- In the presence of antibodies, a blue coloration appears which becomes yellow after addition of stop solution.
- In the absence of antibodies, no coloration appears. The microplate is read at wave length of 450 nm.

The test is valid if:

i. the mean optical density (OD) value of the positive control is greater than 0.250

ii. the ratio of the mean values of the positive and negative controls is greater than 3.

Interpretation of results

Sample to positive (S/P) ratio for each sample and antibody titer was calculated as follows:

S/P ratio

$S/P = OD_{sample} - OD_{NC} / OD_{PC} - OD_{NC}$

Where NC = negative control and PC = positive control

Antibody titer

Log_{10} (titer) = 1X log_{10} (S/P) + 3.630

Titer = $10^{log10\ (titer)}$

Results are interpreted as follows:

S/P value	ELISA Antibody titer	IBV immune status
S/P ≤ 0.2	Titer ≤ 853	Negative
S/P > 0.2	Titer > 853	Positive

Appendix B: The protocol for RNA extraction

Materials

i. Tracheal and cloacal swab samples

ii. Ethanol

iii. 1.5 ml micro centrifuge tubes

iv. Sterile, RNase-free pipet tips (with aerosole barriers for preventing cross-contamination)

v. Micro centrifuge (with rotor for 1.5 ml and 2 ml tubes)

vi. Kit contents:

a. QIAamp® Mini Spin Columns	50
b. Collection Tubes (2ml)	200
c. Buffer AVL (Lyses buffer)	31ml
d. Buffer AW1 (wash buffer;) (concentrate)	19ml
e. Buffer AW2 (wash buffer;)(concentrate)	13ml
f. Buffer AVE (elution buffer;)3x2ml	
g. Carrier RNA (poly A)	310µg
h. Hand book	

Method for QIAamp® RNA extraction of 10 samples as recomended by the manufacturer

Addition of 5.6 mL of buffer AVL and 56 µL of cRNA to a microfuge tube

1. Addition of 560 µl of the mixture to each microfuge tube
2. Addition of 140 µl sample and vortexing for 15 seconds
3. Incubating at room temperature for 10 to 20 minutes
4. Brief centrifuging
5. Addition of 560 µl of absolute ethanol, vortexing for 15 seconds, centrifuging
6. Addition of 630 µl of the mixture to mini column without wetting the rim, centrifuging at 8000rmp for 1 minute
7. Transfer of column to a different tube and discarding the filtrate
8. Repetition of step 6
9. Addition of 500 µl of buffer AWI/ and centrifuging at 8000rmp for 1 minute. Transferring to a 2mL collection tube and discarding filtrate
10. Addition of 500 µl of buffer AWII and centrifuging at 14000rmp for 3 minutes
11. Discarding of collection tube with filtrate and placing the column on new microfuge tube. Centrifuging at 1400rmp for 1 minute
12. Discarding filtrate and transferring to a new microfuge tube followed by addition of 60 µl of elusion buffer; incubating at room temperature for 1 minute
13. Centrifuging at 8000rpm for 1 minute
14. Aliquoting and preserving at -20^0C

Appendix C: Swab samples used for RNA extraction:

1. **TR/B/SK 05**
2. CL/B/SK/05
3. **TR/B/SK 06**
4. CL/B/SK/06
5. **TR/B/SK/23**
6. CL/B/SK/23
7. TR/L/SK/04
8. CL/L/SK/04
9. TR/L/SK/30
10. CL/L/SK/30
11. TR/P/SK/01
12. CL/P/SK/01
13. TR/P/SK/05
14. **CL/P/SK/05**
15. **TR/AF/SK/07**
16. CL/AF/SK/07
17. TR/AF/SK/08

18. CL/AF/SK/08

19. TR/AF/SK/10

20. CL/AF/SK/10

21. TR/AM/SK/06

22. **CL/AM/SK/06**

23. TR/AM/SK/07

24. CL/AM/SK/07,

25. TR/YM/SK/20

26. CL/YM/SK/20

27. TR/YM/SK/22

28. CL/YM/SK/22

29. **TR/YF/SK/16**

30. CL/YF/SK/16

31. TR/B/SK 01

32. CL/B/SK/01

33. TR/P/SK 06

34. CL/P/SK/06

35. TR/P/SK/03

36. CL/P/SK/03

37. TR/L/SK/03

38. CL/L/SK/03

39. TR/YF/SK/02

40. CL/YF/SK/02

41. TR/L/SK/01

42. CL/L/SK/01

43. TR/AF/SK/09

44. CL/AF/SK/09

45. TR/AF/SK/11

46. CL/AF/SK/11

47. TR/AF/SK/12

48. CL/AF/SK/12

49. TR/AM/SK/13

50. CL/AM/SK/13

51. *TR/AM/SK/14*

52. *CL/AM/SK/14*

53. *TR/YM/SK/19*

54. *CL/YM/SK/19*

55. *TR/YM/SK/22*

56. *CL/YM/SK/22*

57. TR/YF/SK/13

58. CL/YF/SK/13

59. TR/B/TB 01

60. CL/B/TB/01

61. TR/L/TB/03

62. CL/L/TB/03

63. TR/AM/TB/03

64. CL/AM/TB/03

65. *TR/B/TB 01*

66. *CL/B/TB/01*

67. *TR/L/TB/01*

68. *CL/L/TB/01*

69. TR/L/TB/03

70. CL/L/TB/03

71. TR/AM/TB/03

72. CL/AM/TB/03

73. TR/AM/GW/03

74. CL/AM/GW/03

75. TR/YM/GW/08

76. **CL/YM/GW/08**

77. TR/YF/GW/01

78. CL/YF/GW/01

79. TR/AM/GW/02

80. CL/AM/GW/02

81. TR/YM/GW/04

82. CL/YM/GW/04

83. TR/YF/GW/05

84. CL/YF/GW/05

85. TR/B/IS/11

86. CL/B/IS 11

87. **TR/AF/IS/04**

88. CL/AF/IS/04

89. **TR/YM/IS/15**

90. **CL/YM/IS/15**

91. TR/YF/IS/12

92. CL/YF/IS/12

93. TR/B/IS/01

94. CL/B/IS/01

95. TR/AF/IS/02

96. CL/AF/IS/02

97. TR/YM/IS/03

98. CL/YM/IS/03

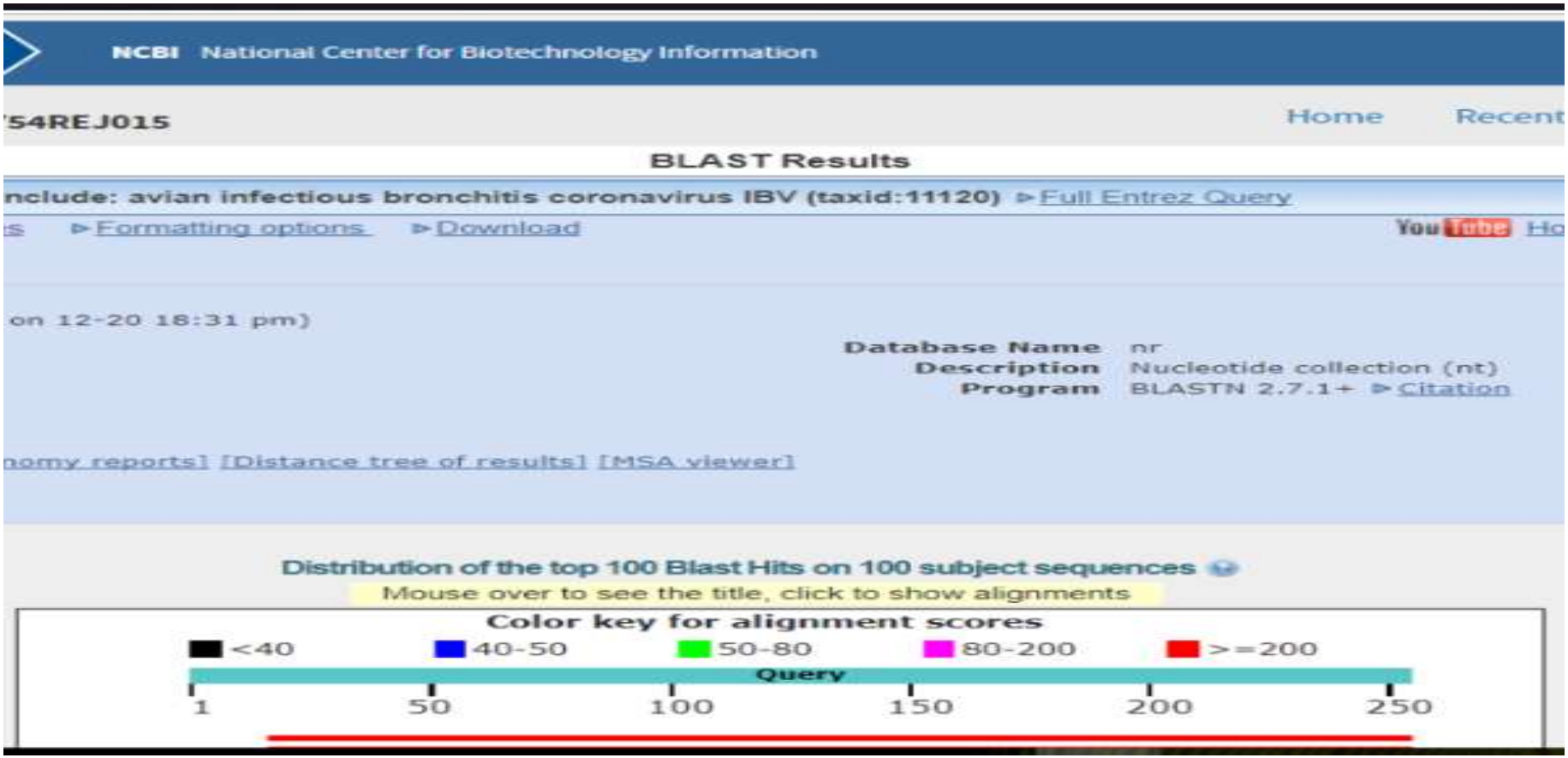
99. TR/YF/IS/05

100. CL/YF/IS/05

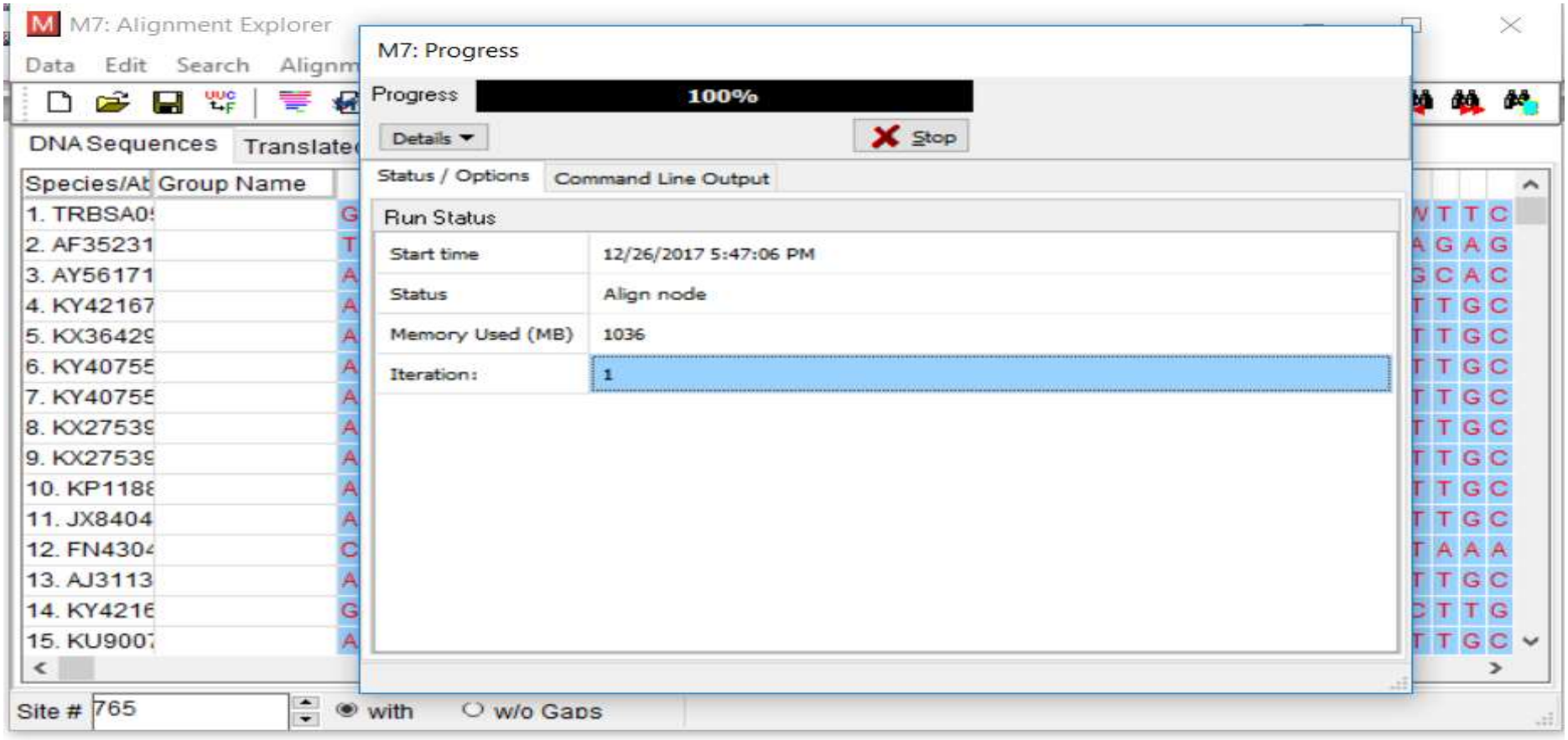
The Italicized codes indicate samples that failed purity and concentration test. The bold codes indicate samples that were PCR positive.

TR= Trachea, CL= Cloaca, B= Broiler, L= Layer, P= Pullet, AF= Adult female, AM= Adult male, YF= Young female, YM= Young male, SK= Sokoto, TB= Tambuwal, GW= Gwadabawa, IS= Isa

Appendix D: Alignment of Sequences



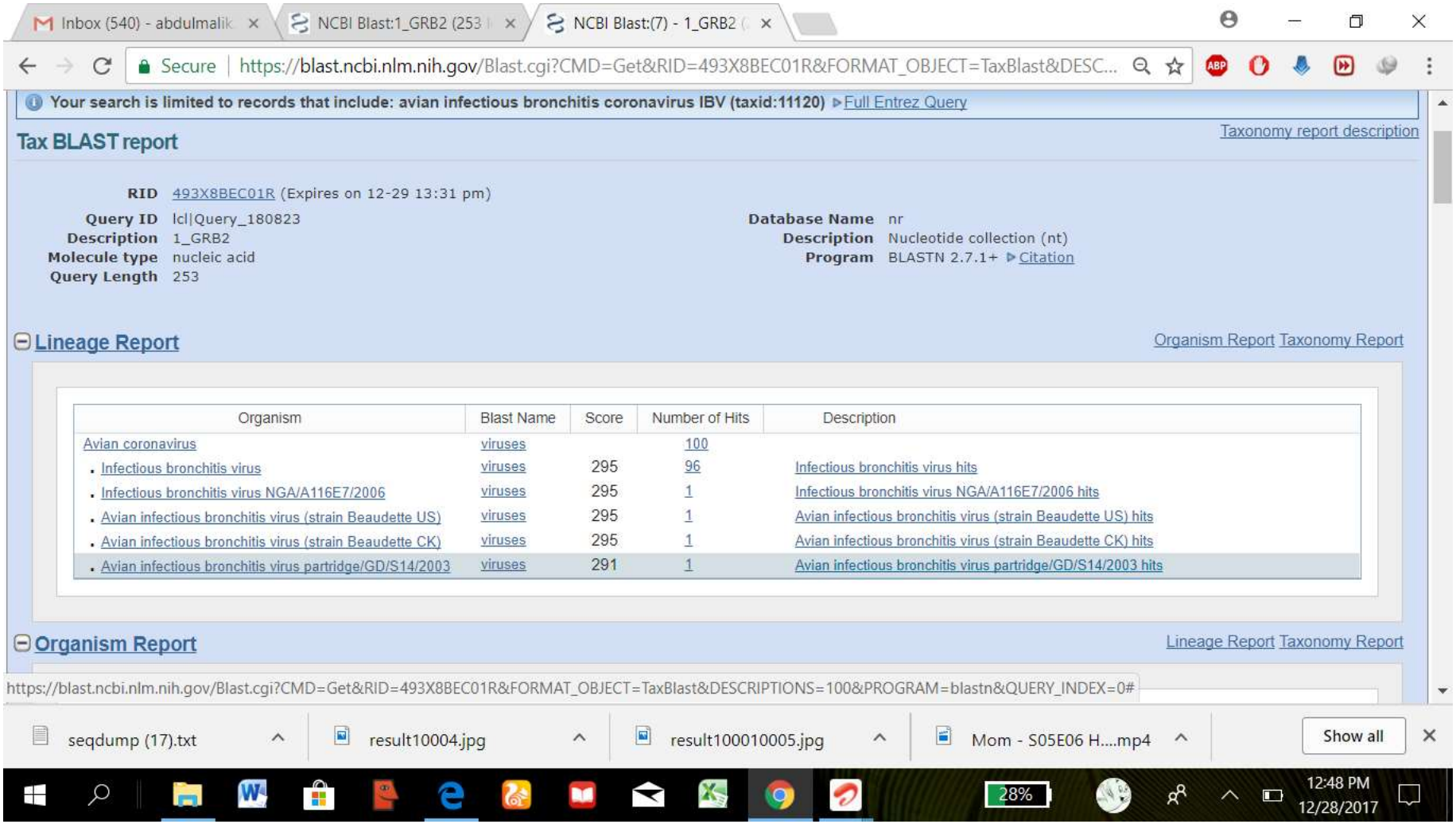
Basic Local Alignment Search output



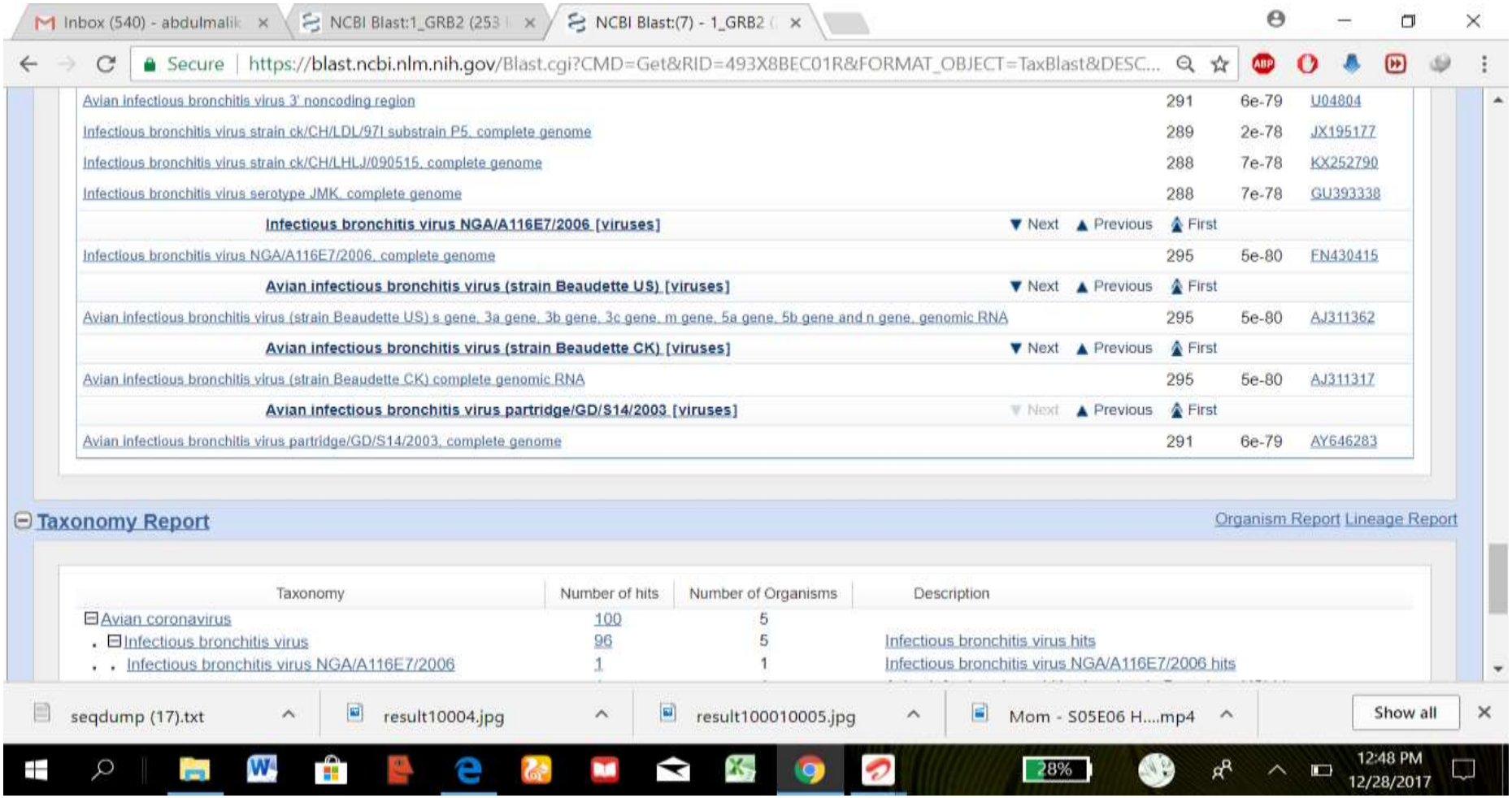
Alignment explorer showing MUSCLE alignment in progress

7 8 9 10 11 12 13 NC

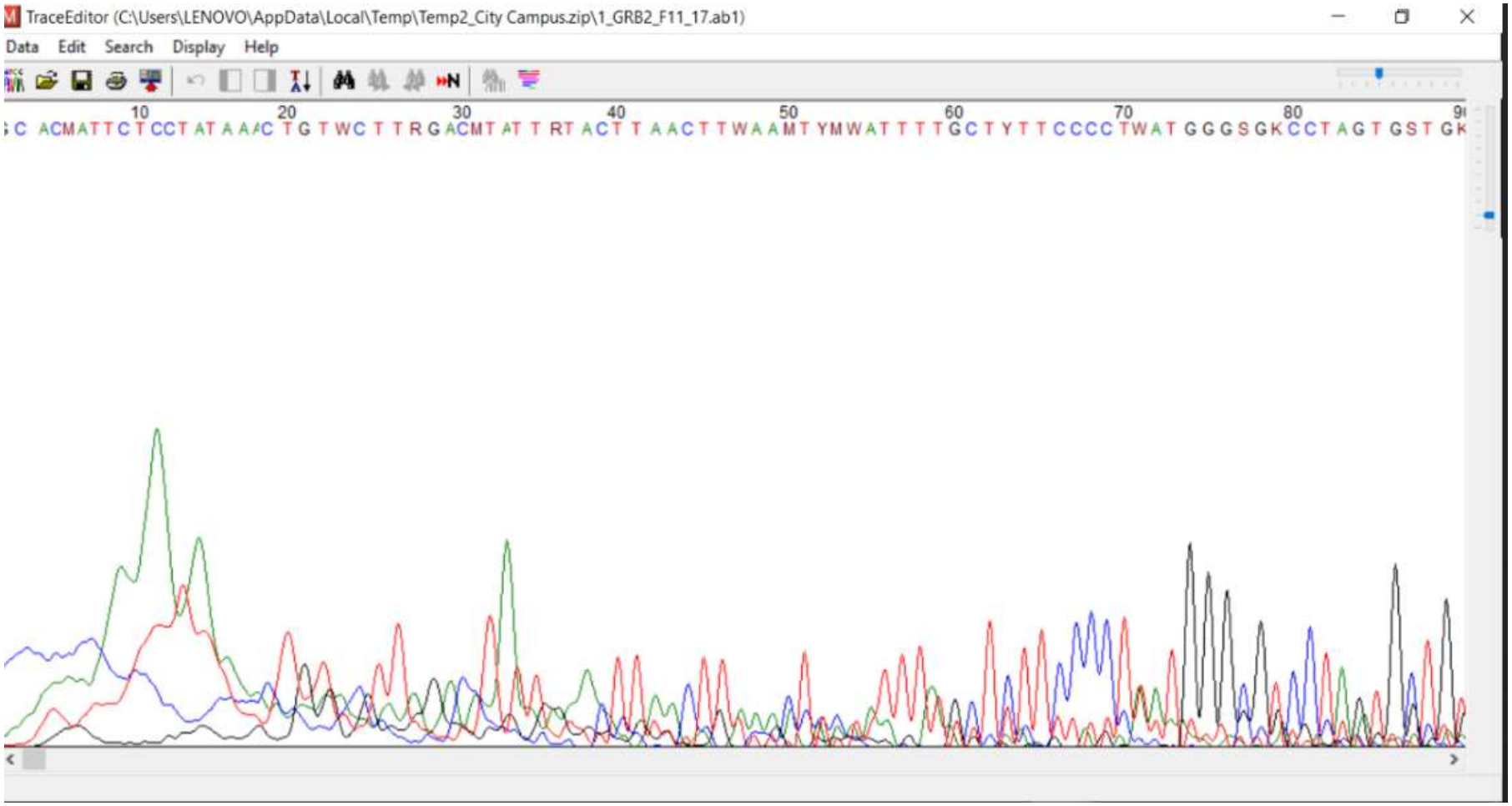
PP2te



Lineage Report



Taxonomy BLAST report



Mega 7 Trace Editor Output