ENTERIC VIRUSES ASSOCIATED WITH RUNTING-STUNTING SYNDROME IN COMMERCIAL BROILERS AND TURKEYS IN SOUTHWESTERN NIGERIA

ADEBOWALE IDRIS ADEBIYI

D.V.M., M.Sc. (Ibadan)

Matric No.: 101565

A Thesis in the Department of Veterinary Microbiology
Submitted to the Faculty of Veterinary Medicine in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN, IBADAN

NIGERIA

FEBRUARY 2021

ABSTRACT

Runting-Stunting Syndrome (RSS) is a transmissible disease associated with different enteric viruses in poultry worldwide. This syndrome causes considerable economic losses due to hatchery condemnations, poor feed conversion, decreased body weight and poor uniformity at slaughter. In Nigeria, these conditions are common in commercial poultry production but little is known about the role of enteric viruses in their aetiology. The presence of enteric viruses [Chicken Astrovirus (CAstV), Avian Nephritis Virus (ANV), Turkey Astroviruses (TAstV-1 and TAstV-2), Avian Reovirus (ARV), Avian Rotavirus (AvRV), Chicken Parvovirus (ChPV) and Fowl Adenovirus (FAdV)] was therefore investigated in commercial broilers and turkeys with RSS in Ogun, Osun and Oyo States, Nigeria.

Using purposive sampling method, 261 blood and intestinal content samples each were collected between November, 2017 and April, 2018 from 158 Day-Old Chicks (DOCs), six 14-week-old chickens and 97 turkey poults. The birds comprised 164 commercial broilers (Ogun 67, Osun 20, Oyo 77) and 97 turkeys (Osun 14, Oyo 83) with signs of RSS from poultry farms (n=10) and hatcheries (n=6). Sera were screened for CAstV and ANV antibodies using immunofluorescence assay. Suspensions (10%) of intestinal contents in phosphate buffered saline were processed for virus isolation in chicken embryo liver cells and identified by Electron Microscopy (EM). Detection and quantification of viral nucleic acid was performed by conventional and real-time Polymerase Chain Reaction (PCR) or Reverse Transcriptase-PCR (RT-PCR). Amplicons obtained were sequenced bi-directionally and phylogenetic analysis was performed with a software. Data were analysed using one-way ANOVA ($\alpha_{0.05}$).

Seropositivity was 7.9% and 3.1% for CAstV in broilers and turkeys, respectively but 0% for ANV in both. Four isolates were obtained: three confirmed as FAdV in DOCs by EM and conventional PCR, and one as ARV by RT-PCR in 14-week-old chickens. Real-time RT-PCR and PCR assays detected CAstV RNA ($\log_{10}$ 2.2-8.0) in 100.0% (164/164), ANV RNA ($\log_{10}$ 2.8-4.9) in 4.9% (8/164) and ChPV DNA ($\log_{10}$ 2.6-3.2) in 3.7% (6/164) of chickens sampled. Five DOCs and all 14-week-old chickens were positive for both CAstV and ANV. In turkeys, 81 out of 97 were positive for CAstV RNA ($\log_{10}$ 2.3- 6.2). All the samples tested negative for AvRV, TAstV-1 and TAstV-2. Multiple alignment of CAstV nucleotide sequences showed high similarity (98.0-100.0%), while phylogenetic analysis revealed they belonged to CAstV Bi clade. Furthermore, ANV (80.0% homology with ANV-2 strain), ARV (83.0% homology with a German strain), ChPV (95.0% identity with enteric parvoviruses) and FAdVs (99.0% and 97.0% identities with serotypes 4 and 5, respectively) were identified.

The association of enteric viruses with runting-stunting syndrome in southwestern Nigeria was established. High detection rate of group Bi CAstV and low occurrence of ANV, ARV, ChPV and FAdV suggested that CAstV was strongly associated with hatchery condemnations and runting-stunting syndrome in commercial broilers and turkeys. Vaccination of commercial breeder birds to prevent vertical transmission of these viruses is recommended.

Keywords: Enteric viruses, Runting-Stunting Syndrome, Commercial breeder birds

Word count: 468

CERTIFICATION

I certify that this work was carried out by

Adebowale Idris **ADEBIYI**

in the Department of Veterinary Microbiology, University of Ibadan, Ibadan, Nigeria under my supervision

……..

Professor Daniel Oladimeji OLUWAYELU
DVM, M.Sc., PhD (Ibadan), FCVSN
Department of Veterinary Microbiology,
University of Ibadan, Ibadan, Nigeria

DEDICATION

This work is dedicated to the loving memory of my late parents. You encouraged me to always make a positive difference and your words of support linger on.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor, Prof. D.O. Oluwayelu for his consistency in monitoring the course of this project. Thank you for your guidance and patience.

I am very grateful to my host at the Avian Virology Laboratory, Agri-Food and Bioscience Institute (AFBI), Belfast, United Kingdom, Dr. Victoria Smyth for granting access to her laboratory facilities and being a friend. Also, my special gratitude goes to James Trudgett, Jonathan MacGraw, David Brennan, Paula Tregaskis and Claire Carlisle for their great support.

My appreciation goes to Dr. A.B. Ayinmode for his immense support and kind words that always lift my spirit. Special thanks to Dr. Oyeduntan Adediran; for making my first interaction with research interesting and being a mother to me. I am grateful to Messrs Gboyega Aluko and Paul Ojewunmi, Drs Shola Ogunsanya, Oladipo Omotosho, Emmanuel Umeh, Ismail Adeyemo and Ayoyimika Ajayi for your various assistances that saw me through this work. I am also grateful to all the poultry farmers and hatchery personnel for helping out with sample collection.

My sincerely appreciation to the Postgraduate School, University of Ibadan, for the scholarship award granted me. I also appreciate Biomatters limited, USA for the free subscription to use the Geneious® bioinformatics software. I also appreciate Michael, Nathalie, Talitha and Yan Taylor for making my stay at their house memorable, truly you are my family.

My profound appreciation to my siblings; Aderonke Aror, Olatunde and Abimbola Adebiyi, Adebukola Aderounmu, Adebola and Bukola Adebiyi, Adeola Alao, Olatunji and Tolulope Alao and Mofiyinfoluwa and Oluwaseun Adewolu. You all have been my support system. I also appreciate my wonderful mothers; Olabisi Adebiyi and Theresa Alao for their prayers and support that saw me through trying times in the course of this programme.

To my jewel of inestimable value, Olamide, thank you for never allowing the sun ever frown. Truly, you know how to bring out the best in me and never allowed the thoughts of “throwing in the towel” an option to ever consider. To my amazingly

wonderful children, Mololuwa and Iyinoluwa, Thank you for your support, and to answer your question why I always collect “poo poo”; this is the reason.

To the everlasting, ever glorious and ever reliable Father; my very present help in the time of need, all I can say is that “Lord, you are forever faithful”

……..
Adebowale Idris Adebiyi

TABLE OF CONTENTS

Page Title	Page
Title page	i
Abstract	ii
Certification	iii
Dedication	iv
Acknowledgements	v
Table of Contents	vii
List of Tables	xv
List of Figures	xvii
List of Plates	xix
List of Abbreviations	xx
CHAPTER ONE: Introduction	1
1.1 Research Questions	2
1.2 Rationale for the Study	2
1.3 Aim	3
1.4 Objectives	4
CHAPTER TWO: Literature Review	5
2.1 History of Poultry Production	5
2.1.1 Poultry Health and Production	6
2.2 Enteric Viruses	6
2.2.1 Virus Entry and Shedding	6

2.3 Runting-Stunting Syndrome 8

2.4 Enteric Viruses Associated with Runting-Stunting Syndrome 9

2.4.1 Astroviruses 10

2.4.1.1 History and Classification 12

2.4.1.2 Mamastrovirus 13

2.4.1.3 Avastrovirus 13

2.4.1.4 Chicken Astrovirus 14

2.4.1.5 Avian Nephritis Virus 15

2.4.1.6 Turkey Astrovirus 15

2.4.2 Properties of the Viral Particles 16

2.4.3 Replication of Virus 16

2.4.4 Virus Distribution 17

2.4.5 Virus Diagnosis 18

2.4.6 Susceptibility of Astroviruses to Physical and Chemical Agents 18

2.4.7 Pathogenesis and Epidemiology 18

2.4.8 Disease Management 19

2.5 Chicken and Turkey Parvovirus 19

2.5.1 History and Classification 19

2.5.2 Virus Characteristics 20

2.5.3 Virus Replication 22

2.5.4 Pathogenesis and Epizootiology 22

2.5.5 Diagnosis 23

2.5.6 Prevention and Control 24

2.6 Avian Rotavirus 24

2.6.1 Virus, Classification and Genome Organization 25

2.6.2 Virus Replication 26

2.6.3 Pathogenesis and Epizootiology 27

2.6.4 Diagnosis, Prevention and Control 27

2.7 Avian Reovirus 28

2.7.1 Virus Properties 29

2.7.2 Replication of Orthoreoviruses 29

2.7.3 Pathologic Process and Epidemiology of Orthoreoviruses 30

2.7.4 Diagnosis 30

2.8 Fowl Adenovirus 31

2.8.1 History and Classification of Adenoviruses 31

2.8.2 Properties of Adenoviruses 34

2.8.3 Replication of Virus 35

2.8.4 Aviadenovirus 35

2.8.5 Pathogenesis and Epizootiology 35

2.8.6 Diagnosis 36

2.8.7 Prevention and Control 36

2.9 Identification Considerations for Enteric Viral Pathogens of Poultry 37

2.10 Surveillance and Disease Management 39

2.10.1 Disease Surveillance 39

2.10.2 Control of Disease by Means of Sanitation 40

2.10.3 Use of Quarantine as a Tool for Control of Disease 40

2.10.4 Disease Control through Vaccination 41

2.10.5 Effects of Animal Rearing Method on Control of Diseases 42

2.11 Economic Importance of Runting-Stunting Syndrome in Poultry in Nigeria 43

2.12 Runting-Stunting Syndrome in Poultry in Nigeria 43

CHAPTER THREE: Materials and Methods 45

3.0 Ethical Approval 45

3.1 Sample Descriptions 45

3.1.1 Sample Location 45

3.1.2 Sources of Collected Samples 45

3.1.3 Design and Period of Study 49

3.1.4 Sample Collection, Storage and Transportation 49

3.1.4.1 Blood Samples 49

3.1.4.2 Intestine Contents 53

Section One

3.2 Serodetection of Astroviruses in Commercial Broiler Chickens and Turkey Poults with Runting-Stunting Syndrome 54

3.2.1 Indirect Immunofluorescent Assay 54

3.2.1.1 Cleaning and Sterilization of Multispot Slides 54

3.2.1.2 Preparation of Phosphate Buffered Saline 54

3.2.1.3 Tissue Culture Preparations of Antigen infected Cells on Multispot Slides 54

3.2.1.4 Testing of Serum by Indirect Immunofluorescent Assay 55

3.2.2 Statistical Analysis 57

Section Two

3.3 Isolation of Enteric Virus Associated with Runting-Stunting Syndrome in Commercial Broilers and Turkeys 59

3.3.1 Preparation of Reagents 59

3.3.2 Preparation of 10% (w/v) Intestinal Content Suspension 59

3.3.3 Cell Culture Preparation 60

3.3.3.1 Preparation of Cell Culture Reagents 60

3.3.4 Preparation of Primary Chick Embryo Liver Cell Cultures 61

3.3.5 Virus Isolation 64

3.3.6 Identification by Negative Contrast Electron Microscopy 66

3.3.6.1 Preparation of Sample Suspension 66

3.3.6.2 Negative Staining of Test Samples and Identification 66

3.3.7 Statistical Analysis 66

Section Three

3.4 Molecular Detection of Enteric Viruses Associated with RSS in Commercial Broilers and Turkeys 68

3.4.1 Nucleic Acid Extraction 68

3.4.2 Chicken Astrovirus and Avian Nephritis Virus Capsid Protein RT-PCR Assays 69

3.4.3 Detection of Non-Structural Protein of Avian Rotavirus 72

3.4.4 Detection of the Sigma C Protein of Avian Reovirus 72

3.4.5 Detection of Polymerase Gene of Turkey Astroviruses 1 and 2 73

3.4.6 Detection of Non-Structural Protein Gene of Parvoviruses 73

3.4.7 Detection of Hexon Gene of Fowl Adenovirus 76

3.4.8 Evaluation of Conventional RT-PCR and PCR Products 76

3.4.8.1 Preparation of Agarose Gel 76

3.4.8.2 Gel Electrophoresis 76

Section Four

3.5 Quantification of Chicken Astrovirus, Avian Nephritis Virus and Chicken Parvovirus by real-time RT-PCR and PCR 78

3.5.1 Chicken Astrovirus and Avian Nephritis Virus 78

3.5.2 Chicken Parvovirus 82

Section Five

3.6.1 Sequencing of obtained enteric RNA and DNA viruses 86

3.6.1.1 Gel excision and purification 86

3.6.1.1a Gel excision and dissolution 86

3.6.1.1b Purifying DNA using a centrifuge 86

3.6.1.2 Sequencing of DNA 87

3.6.2 Analysis of sequences 88

3.6.3 Phylogenetic analysis 88

3.6.4 Gene sequence 88

CHAPTER FOUR: Results and Discussion

Section one

4.1 Serodetection of astroviruses by indirect immunofluorescent assay 90

Section two

4.2.1 Isolation of enteric viruses in chicken embryo liver cells 94

4.2.2 Identification of enteric viruses using transmission electron microscopy 94

Section three

4.3 Detection of enteric viruses using conventional RT-PCR and PCR 99

4.3.1 CAstV and ANV capsid protein RT-PCR assays 99

4.3.2 Avian rotavirus RT-PCR 99

4.3.3 Avian reovirus RT-PCR 99

4.3.4 Turkey astroviruses 1 and 2 RT-PCR 103

4.3.5 Parvovirus non-structural protein gene PCR 103

4.3.6 Fowl adenovirus hexon gene PCR 103

Section four

4.4 Quantification of CAstV, ANV and ChPV 106

4.4.1 CAstV and ANV RT-qPCR 106

4.4.2 ChPV qPCR 112

Section five

4.5 Determination of phylogenetic relatedness between detected enteric Viruses with reference strains 114

4.5.1 Nucleotide sequence alignment 114

4.5.2 Phylogenetic analysis 120

4.6 Discussion 130

CHAPTER FIVE: Summary and Conclusions

5.1 Summary 144

5.2 Conclusions 144

5.3 Contributions of this Study to Scientific Knowledge 144

References 146

Appendix 159

Ethical Approval for the Study 157

LIST OF TABLES

Table	Page
3.1 Collection sites of blood samples from broiler chickens and turkeys	47
3.2 Distribution of samples based on collection source	48
3.3 AffinityScript One-Step RT-PCR Kit recommended assay concentration	70
3.4 Primers and nucleotide sequences applied to the identification of enteric viruses	71
3.5 Hot Start Taq PCR Kit recommended assay concentration	75
3.6 Primers and nucleotide sequences for CAstV and ANV RT-qPCR assays	80
3.7 RT-qPCR Master mix for ANV and CAstV assay	81
3.8 Real-time PCR Master Mix for ChPV assay	83
3.9 Primers and nucleotide sequences for ChPV qPCR assay	85
4.1 Serodetection of avian astroviruses in commercial broiler chickens and turkeys	93
4.2 TEM detection of fowl adenovirus in cell culture isolates	98
4.3 Capsid RT-PCR assays for CAstV and ANV detection in commercial broiler chickens and poults	101
4.4 Evaluation of CAstV RNA by Real time RT-PCR in commercial broilers	108
4.5 Evaluation of CAstV RNA by Real time RT-PCR in commercial turkeys	109
4.6 Evaluation of ANV RNA by Real time RT-PCR in commercial broilers	111
4.7 Evaluation of ChPV DNA by Real time PCR in commercial broilers	113
4.8a CAstV capsid sequences for phylogenetic investigation	115
4.8b ChPV sequences for phylogenetic investigation	116
4.8c ARV sequences for phylogenetic investigation	117

4.8d FAdV sequences for phylogenetic investigation 118

4.8e ANV sequences for phylogenetic investigation 119

LIST OF FIGURES

Figure	Page
3.1 Map of Nigeria showing collection sites	46
3.2a Runted day old broiler chick with signs of paralysis and ruffled feather	50
3.2b Runted poults with poor feather development and paralysis	51
3.2c Stunted 14-week old broilers	52
3.3 The MagNA Pure 96 (Roche, UK) robotic workstation	68
4.1 Immunofluorescent staining (FITC) with CAstV 11672-infected cells showing intracytoplasmic inclusion bodies	91
4.2 Chicken embryo liver cells with (A) uninfected (control) cells, (B) infected cells with presentation of cell sloughing as well as death	95
4.3 Adenovirus-like particle visualized with transmission electron microscopy using negative staining	96
4.4 Unknown virus-like particle visualized with transmission electron microscopy using negative staining	97
4.5 Capsid gene RT-PCR assays for CAstV and ANV in commercial broiler Chickens and poults	100
4.6 RT-PCR detection of avian reovirus (ARV) in commercial broilers	102
4.7 PCR detection of chicken parvovirus (ChPV) in commercial broilers	104
4.8 PCR detection of fowl adenovirus (FAdV) in commercial broilers	105
4.9 Real-time amplification of CAstV RNA in commercial broilers and turkeys	107
4.10 Real-time amplification of ANV RNA in commercial broilers and turkeys	110
4.11 Phylogenetic relationship among Nigerian and reference CAstV strains established by sequences of the capsid protein nucleotides	121

4.12 Phylogenetic relationship among Nigerian and reference strains of ARV established by nucleotide sequence of σ C protein 123
4.13 Phylogenetic relationship among Nigerian and reference ChPV strains established by sequences of the non-structural protein nucleotides 125
4.14 Phylogenetic relationship among Nigerian and reference FAdV strains according to hexon gene sequence analysis 127
4.15 Phylogenetic relationship among Nigerian and reference ANV strains based on nucleotide sequence of capsid protein gene 129

LIST OF PLATES

Plate	Page
2.1 Transmission electron (negative contrast) micrograph of adenovirus-2 particle	32
2.2 The diverse categorizations of adenovirus genomes in the 4 established genera	33
3.1 Multispot slides with test samples placed on staining rack in humidified chamber	56
3.2 Slicing of liver samples from specific pathogen free (SPF) chicken embryos	63
3.3 Inoculation of 10% tissue homogenates onto cell culture	65

LIST OF ABBREVIATIONS

RSS: Runting-stunting syndrome

CAstV: Chicken astrovirus

ANV: Avian nephritis virus

ChPV: Chicken parvovirus

TAstV: Turkey astrovirus

ARV: Avian reovirus

AvRV: Avian rotavirus

FAdV: Fowl adenovirus

SPF: Specific-pathogen-free

ORF: Open reading frame

ICTV: International Committee on Taxonomy of Viruses

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

RT-PCR: Reverse transcriptase polymerase chain reaction

VP: Virus protein

IFAT: Immunofluorescence assay test

BLAST: Basic Local Alignment Search Tool

IIF: Indirect immunofluorescence assay

FITC: Fluorescein isothiocyanate

PBS: Phosphate buffered-saline

EM: Electron microscopy

W/V: Weight per volume

CPE: Cytopathic effect

CELC: Chicken embryo liver cell culture

RT-qPCR: Real time (quantitative) reverse transcriptase polymerase chain reaction

qPCR: Real time (quantitative) polymerase chain reaction

6-FAM: 6-Carboxyfluorescein

MGB: Minor groove binder

NCBI: National Center for Biotechnology Information

DEPC: Diethyl pyrocarbonate

TAE: Tris-acetate ethylene diamine tetra- acetic acid

MEGA: Molecular Evolutionary Genetics Analysis

TEM: Transmission Electron Microscopy

AFBI: Agri-Food and Biosciences Institute

NSP: Non structural protein

σ C: Sigma C

FAOSTAT: Food and Agriculture Organisation Statistics

IBV: Infectious Bronchitis Virus

LMH: Chicken hepatocellular carcinoma cell line

D-PBS: Dulbecco's phosphate buffered-saline

Kb: kilo base

CT: cycle threshold

RRM: Ready reaction mix

CHAPTER ONE

INTRODUCTION

Poultry has progressively emerged as a significant financial contributor in several developed and less developed economies as they have become a major source of animal protein all over the world. In addition, egg production, increased taste for chicken, ease of sales and short production-sales period have increasingly added to poultry farming popularity. However, infections involving the gastrointestinal tract are of immense consequence in poultry production affecting feed uptake by birds, reduced productivity and increased predisposition to secondary infections (Smyth, 2017). Factors such as microbes and poor rearing system are capable of negatively affecting the gut health making enteric infections widespread in poultry (Barnes and Guy, 2003).

Runting-Stunting Syndrome (RSS) has generally been known as a transmissible microbial disease of chickens with its aetiology remaining uncertain. Chicks are infected virtually after hatch with the condition characterised by poor feather development, retardation of growth, as well as diarrhoea, leading to substantial loss of financial viability in poultry because of decreased body weights, size inconsistency, reduced feed conversions, diminished liveability and increased vulnerability to other infections (Zavala and Barbosa, 2006). Initial reports of RSS were made in the 1970s in the broiler industry and have since gained relatively substantial worldwide appreciation (Olsen, 1977; Nunez *et al.,* 2016; Long *et al.,* 2017). Owing to absence of a recognized aetiology, the description of this condition usually relies on different expressions that include helicopter syndrome, broiler runting syndrome, malabsorption syndrome and spiking mortality (Smyth, 2017). Enteric viruses are a major concern in broiler poultry production because they constitute a major cause of enteritis and growth-related conditions such as runting-stunting syndrome (Dai *et al.,* 2010).

Considerable losses of financial viability owing to the need to dispose less viable birds that failed to thrive or make suitable slaughter weight have been demonstrated in many poultry operations. In addition, enteric viruses of chickens that include calicivirus,

enteric chicken parvovirus (ChPV), avian rotavirus (AvRV), chicken astrovirus CAstV), fowl adenovirus (FAdV), avian nephritis virus (ANV) and avian reovirus (ARV) linked to RSS as solitary or co-infections maintain immense research attention worldwide (Awe *et al.,* 2015; Kang *et al.,* 2018). Interestingly, some of these pathogens such as astroviruses, reovirus and rotavirus have also been noticed in apparently healthy birds thus complicating their exact connections with this disease condition (Devaney *et al.,* 2016). Thus, the overall impact of these enteric viruses on poultry health and economics as well as their exact role in RSS is hitherto not appreciated in detail (Smyth, 2017). Based on these foregoing observations in poultry, the following research questions were proposed:

1.1 RESEARCH QUESTIONS

i. Can enteric viruses be established in commercial broilers and turkeys with runting-stunting syndrome in Ogun, Osun and Oyo States, Nigeria?
ii. What are the genotypes of the detected enteric viruses associated with RSS in the study area?
iii. Is there any relationship between strains detected in chickens and turkeys with RSS in the study area?

1.2 RATIONALE FOR THIS STUDY

There have been varying extents of devastations in poultry production by viral enteric infections. There is a propensity of these viral infections to have an effect principally on young birds; nonetheless, these diseases could take place at any age, as such increasing susceptibility to further infections, reduce competence for feed adaptation as well as protract slaughter weight attainment (Barnes and Guy, 2003; Saif, 2008). Viruses such as chicken calicivirus, astroviruses, AvRV, ChPV, ARV, FAdV and IBV in turkeys and chickens have been related to enteric diseases. These enteric pathogens have been of a major concern in broiler poultry production in recent years since they constitute a major cause of intestinal infections and growth-related conditions such as RSS (De la Torre *et al.,* 2018). Moreover, infection with these viruses is of considerable consequences in enteric microbial diseases including RSS.

Birds are affected with RSS virtually after hatch characterised by poor feather development, retardation of growth, as well as diarrhoea, leading to substantial loss of financial viability in the meat-type poultry industry. Young chickens, mainly broiler chickens and turkey poults (Barnes and Guy, 2003) are most susceptible. Furthermore, chickens and turkeys without symptoms of enteric disease have been reported to be positive for these viruses (Devaney *et al.,* 2016), indicating that they could shed this pathogen while not presenting signs of infection. Such birds regarded as apparently healthy reservoirs represent means of prospective disease infection to clean birds.

In Nigeria, nearly 10% of the agricultural gross domestic product is contributed by the livestock segment (FAOSTAT, 2014); out of which poultry production appreciably add to this number. Despite this important contribution by this vital subdivision, poultry production is immensely inhibited by contagious diseases making the industry less financially viable. Although occurrences of growth retardation, feed inefficiently converted and diarrhoea/enteritis are common in commercial poultry operation in Nigeria, they have not been linked to enteric viruses. In spite of reports linking enteric viruses with RSS or poor performance of poultry elsewhere (Devaney *et al.,* 2016; Smyth, 2017; De la Torre *et al.,* 2018), apart from report in broiler chicks of RSS based on histopathological and microbial identification (Ighodalo and Akpavie, 2008) as well as detection of CAstV and ANV in apparently healthy indigenous chickens (Oluwayelu *et al.,* 2011; Oluwayelu and Todd, 2012), there is little or no information on enteric viruses associated with RSS or poor poultry performance in Nigeria. Hence, in order to achieve improved comprehension of the health and disease dynamics as it affects the Nigerian poultry industry as well as sustain this sector of approximately two hundred million birds (FAOSTAT, 2014); there is a need to conduct surveillance for enteric viruses associated with RSS in commercial broilers and turkeys.

1.3 AIM

This study was carried out to investigate the enteric viruses related to runting-stunting syndrome in commercial broilers and turkeys in Ogun, Osun and Oyo States, Southwestern Nigeria

1.4 OBJECTIVES

The specific objectives of this study are to:

1. Detect and quantify enteric viruses associated with RSS in commercial broilers and turkeys using molecular techniques
2. Determine phylogenetic relatedness between the detected enteric viruses in this study with reference strains identified elsewhere
3. Perform serodetection of astroviruses using immunofluorescence assay
4. Isolate enteric viruses using cell culture and determine their ultrastructure using transmission electron microscopy

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY OF POULTRY PRODUCTION

It is believed that poultry production started in Asia over three centuries ago. Although some records propose that chickens were raised around 3200 BC, archaeological verification only goes back to about 2000 BC (Gillespie and Flanders, 2009). Chickens are considered to originate from India, with the red jungle fowl as an ancestor to today's modern chicken. The breeding of chickens in confinement dates back to at least 1400 BC in Egypt (Gillespie and Flanders, 2009). However, intensive poultry production only began in the 20th century. Indeed, the past ten decades have seen a remarkable development, mainly in the production of chickens and eggs, turkeys, ducks, and geese (Daghir, 2008). Additionally, the advent of vaccination for conditions such as Marek's disease, in addition to significant improvements in nutrition and breeding paved way for rapid development of this industry since the late 1960s (Gillespie and Flanders, 2009). By the early 1980s, breeding complexity increased greatly because of carcass and meat yield requirements and continuing improvement in feed conversion and livability. Therefore, variables such as breeding value estimation, feed conversion, meat yield, and disease resistance were important factors considered in selection practices (Jez *et al*., 2011). Moreover, unique selection indices or markers have been created, considering production, health and well-being traits. Also, welfare concerns in developed countries have also resulted in new production standards. About 75% of poultry production in the world is carried out in intensive operations using confinement systems (Jez *et al*., 2011). Difficulties in maintaining a cold chain, a traditional consumer preference for live poultry, and the lack of organization of industries are limiting efficiency and profitability in many developing countries (Daghir, 2008).

2.1.1 POULTRY HEALTH AND PRODUCTION

Chicken production is, by far, the largest source of poultry meat in the world. It is mainly concentrated in North America, Latin America, and Asia. Similarly, turkey meat production is about fifteen times smaller than chicken production, with over ninety percent of such production concentrated in the Americas and Europe. Although production in Africa is increasing, the size of the industry and its growth are in no relation with the size and growth of its human population (FAOSTAT, 2014).Considerable increases have been attained in various sectors of poultry production which has been further enhanced by improvement in nutrition, disease prevention and control, rearing and breeding management. Despite these advancements, the unpredictable course of poultry disease is still of great concern (Saif, 2013).

Elimination or reduction of infectious diseases is maintained by adequate bio-security measures further sustained by regular diagnostic and poultry health monitoring. This close-up system minimizes disease spread as well as allows possible rapid intervention in new episodes of diseases (Jez *et al*., 2011). Conversely, in poultry productions with little or no bio-security measures, particularly common in less-developed economies, further worsened by inadequate personnel and diagnostic capabilities, do not allow timely intervention in disease management. Therefore, there are high chances of the widespread emergence of diseases causing severe loss of resources and financial viability (Jez *et al*., 2011).

2.2 ENTERIC VIRUSES

2.2.1 VIRUS ENTRY AND SHEDDING

The ingestion of contaminated feed or water serves as the main source of enteric viral infections. These viruses principally replicate in the intestinal tract via their direct transportation or virus progenies from infected cells (Kasamatsu and Nakanishi, 1998). Generally, enteric viruses are resistant to bile and acid and further cosseted by the directional peristaltic motion in adults as well as neutralizing effect of milk in the gut of young animals that shield these viruses during movement allowing the viral particles gain access to susceptible epithelial cells (Cann, 2008). On the other hand, the infectivity of

some enteric viruses is essentially improved by exposure to proteolytic enzymes in the gut (Saif, 2013).

Astroviruses, calicivirus, torovirus as well as rotavirus now enjoy being recognized as foremost causative viral agents of animal diarrhoea, whereas great majority of intestinal infections caused by enteropathogens such as adenoviruses remain asymptomatic (Cann, 2008). Viraemic spread of enteropathogens such as parvoviruses to epithelia cells within the intestine lead to diarrhoea and the contamination of the environment by these viral enteropathogens is directly proportional to the volume of the virus shed particularly in fluidic faeces and further enhanced by resistance of these viruses to inactivation by ecological conditions (Saif, 2013).

The families of enteric viral pathogens include the following; *Coronaviridae, Parvoviridae, Astroviridae, Caliciviridae, Reoviridae* (genera *Rotavirus* and *Reovirus), Adenoviridae* and *Picornaviridae* (genus *Enterovirus).* Typically, they are the source of the majority of the primary injuries to the gastrointestinal region of chicks that present other microbes with the environment to reproduce and infiltrate cells, thus promoting more injuries (Kuss *et al*., 2011). Consequently, there is adhesion of bacteria film on the villi at some stage in a number of enteric diseases. Hence, countering resulting microbial involvement is perhaps the basis for the observed efficacy of antimicrobial management of a number of enteropathies activated by viruses in chicks. Furthermore, study by Kuss *et al*. (2011) showed interactions with gut bacteria promote viral enteric infection.

The financial importance of enteric virus diseases in poultry production varies from inconsequential economic outcome to substantial losses of financial viability (Long *et al*., 2017). Young growing birds are primarily impinged on by enteric microbial diseases; nevertheless, all age groups may be involved, thus increasing propensity for new infections, reducing competence for feed adaptation as well as protracted slaughter weight attainment (Saif, 2008). The increasing insight that microbes apart from bacteria should be an essential aetiology of enteric infections coupled with availability of diagnostic tools has inspired investigations of other contagious aetiologies particularly viruses (Smyth, 2017).

Earlier studies have shown significant findings of the occurrence of virus diversities in intestinal tract of growing birds (De la Torre *et al*., 2018; Kang *et al*., 2018). These discoveries drew attention to the need to segregate the viruses to make possible

investigation of the roles played in enteric infections. Accessibility of specific-pathogen-free (SPF) birds has been significant in the investigations (Todd *et al*., 2009). Similarly, the increased accessibility and utilization of molecular approaches has enhanced detection of enteric viruses. Furthermore, the utility of the test has increasingly improved by the ability to remove inhibitors from fecal materials and the use of 'internal controls' for enhanced test precision (Donato and Vijaykrishna, 2017).

The majority of enteric viral infections take place primarily within 21 days of life, but some happen afterward. Although enteric infections of birds may be established immediately after hatch, infection may persist right through all ages of birds with or without observable clinical signs (Veen *et al*., 2017). Clinical pathologies caused by these viruses are particularly not specific; consequently, associating certain viral agent to particular enteric ailments is tricky except laboratory-based identification of the causal agent(s). Besides, there may possibly be diverse disease pathologies as a result of the occurrence of varied combination of viruses (Saif, 2013). The consequence of enteric viral infections in young birds is often runting and/or stunting of the birds resulting in uneven flocks (Saif, 2013). Various viral agents (or combinations of these agents) can cause enteric disease. Enteric diseases that occur in young birds are problematic in poultry production with various viruses linked to enteric diseases of young birds. Some of these viruses are enteropathogens however; the role that other viruses play in enteric disease is yet to be determined.

2.3 RUNTING-STUNTING SYNDROME

Runting-stunting syndrome (RSS) is an infectious microbial disease of poultry that affects birds early after hatch. This disease affects poultry productivity primarily characterised by growth retardation in chicks especially within two to three weeks of life (Rosenberger, 2012) with corresponding reduction of size or alteration of shape of the villi as a result of intestinal cysts development (Kort *et al*., 2013). RSS was first described in the broiler industry during the 1970s with symptoms such as poor feed conversion ratio, undersized birds at hatch (runting) and proventriculitis (Olsen, 1977) and since then gained much description virtually everywhere (Nunez *et al*., 2016, Smyth, 2017). Further researches have described an array of clinical symptoms connected to this malabsorption

disease in poultry such as delayed weight gains, wet and helicopter feathering, leg weakness, enteritis, diarrhoea as well as fatalities in complicated instances leading to loss of financial viability particularly of the meat-type industry (de Wit *et al*., 2011; Smyth, 2017).

Owing to dearth of information on a recognized causative agent, the description of this condition commonly relies on different expressions that include helicopter syndrome, spiking mortality broiler runting and malabsorption syndromes (Smyth, 2017).Currently, for the effective control of the disease, there are no commercially available vaccines, principally as a result of observed intricacy of RSS and multiple aetiologic agents possibilities, hence, development of treatment and control strategies are hindered (Kang *et al*., 2012). Even though ecological and microbial features could be linked as potential contributors toward the disease progression (Kang *et al*., 2012), the most probable aetiologic agents have been pointed out as small, round, non-enveloped viruses (Sellers *et al*., 2010) based on electron microscopy and disease reproducibility using a chloroform-treated, bacteria-free filtrates. Worldwide, occurrences of considerable loss of financial viability experienced by poultry farmers as a result of RSS have led to disposal of poorly developed birds that could not attain slaughter weight (Smyth *et al*., 2017). Of particular research concern is the numerous RNA and DNA enteric viruses linked to RSS as solitary or co-infections which may include astroviruses, chicken parvovirus, avian reovirus, fowl adenovirus and avian rotavirus demonstrated in episodes of deprived performances in birds (Devaney *et al.,* 2016; Radwan *et al*., 2018).

2.4 ENTERIC VIRUSES ASSOCIATED WITH RUNTING-STUNTING SYNDROME

There has been an increasing spotlight on enteric viruses as likely etiology of runting-stunting syndrome in turkeys and chicken with economic as well as health consequences in poultry production. Runting-stunting syndrome infects birds as early as at hatch typified by stall in growth, abnormal featherings as well as diarrhoea, giving rise to reduced liveability and susceptibility to ensuing microbial infections (Smyth, 2017). Proven presentations caused by the various enteric viruses are fairly comparable; thus linking particular condition with certain viruses may be complex except by scientific

identification of the cause (Saif, 2008). Accordingly, the occurrence of different virus combinations in a disease may result in diverse appearance of such disease with a likelihood of low or elevated deaths happening when solitary or several viruses are involved (Todd *et al*., 2009).

Enteric viruses appear usually as source of majority of the crucial damages to the gastrointestinal tracts of growing birds (Koo *et al*., 2013). This affords other microbes the setting to reproduce and break through cells, advancing further damage. Also, during these enteropathies, the surface membrane receptors of the villi may be altered causing a modification of their micro-environment thus allowing opportunistic bacteria adherence and proliferation on the surface of the villi (Ramphal *et al*., 1980). Consequently, the virus-induced mucosal damage may promote gastrointestinal attachments of prospective microbes because of microvilli loss or mucin that serve as normal defence mechanisms. Thus, the resulting microbial involvement is possibly the rationale informing antimicrobial efficacy in viral infections of poultry. However, these rates of infections and deaths are possibly amplified as infection with a combination of microbes rather than a single microbe is involved (Oluwayelu and Adebiyi, 2015). Consequently, treatments with antibiotics after extensive damage had occurred may be of little significance, thus, causing indirect economic losses through repeated uses of antibiotics.

2.4.1 ASTROVIRUSES

Astroviruses are tiny (25-30 nanometres in width) round viral particles naturally spread by ingestion of contaminated feed and water. The name was coined as a result of the observed 5 or 6 barbed exterior protrusions resembling stars during identification (Madeley and Cosgrove, 1975). On the other hand, majority of astroviruses may possibly not display this morphology thus, there may be some reservation concerning the utilization of transmission electron microscopy for identification (Koci *et al*., 2000). Astroviruses are non-enveloped, of single strand and contain ribonucleic acid genome of approximated length of 6.5–7.5 kilobase, a positive polarity and 3 open reading frames (ORF) (Maclachlan and Dubovi, 2011). The synthesis of a sub genomic message during replication, possession of a serine protease and retrovirus-like frame shift signal sequence between RNA-dependent RNA polymerase and nonstructural proteins, distinguish the

replication stratagem of astroviruses from other enteric viruses (Koci *et al*., 2000; Matsui and Greenburg, 2001). In recent times, improved phylogenetic analyses have been sustained by whole genome sequences of numerous avian astroviruses (Smyth *et al*., 2012). These studies demonstrate molecular distinction of astroviruses of avian and mammalian origin with slightly shared sequence similarity in the various gene segments (Matsui and Greenburg, 2001). For instance, an evaluation of genomic conserved area revealed approximately 40% sequence identity in RNA-dependent RNA polymerase between human and avian astroviruses (Willcocks *et al*., 1994).

Astroviruses have been linked with acute gastroenteritis, fatal hepatitis and nephritis in a range of animals (Smyth, 2017). Additionally, astroviruses are among the most frequently associated viral pathogens in runting-stunting syndrome in poultry (Smyth *et al*., 2013; Kang *et al*., 2018). Conventionally, astroviruses nomenclature is based on the animal infected however, species cross-over has been observed such as the detection of avastroviruses of chickens in turkeys (Smyth, 2017). The detection of astroviruses in poultry has significantly improved in recent years, likely as a result of better diagnostic tests and enhanced surveillance.

Infections are commonly observed in poultry with a range of different strains of chicken and turkey avastroviruses. Therefore, diagnostic tests should be able to detect and differentiate among diverse genotypes. There are two common categories for detailed assessment of virus diseases: (1) expression of the existence of viral antibody as well as (2) proves of the existence of infectious virus, virus antigen, or viral nucleic acid. Despite the fact that countless conventional techniques remain commonly utilised, the majority of them appear time-consuming to exercise in the least undeviating control on specific clinical case administration. Thus, the main drive in virus assessment improvement is focused on rapid detection procedures that accomplish the fundamentals involving ease, rapidity, affordability, sensitivity and specificity.

Immunofluorescence assay test (IFAT) is a straightforward, sensitive and rapid way for the qualititative and quantitative antibodies assessment in a serum sample. This is based on the theory that particular antigen-antibody reactions can be identified by means of appropriate anti species secondary antibodies labelled by way of a fluorescent marker. For serum antibody testing it is necessary to have a supply of antigen infected cells in a

suitable form for either small or large scale testing (AFBI, 2012). The test may be carried out using fixed antigen-infected cells cultured on multispot glass slides coated to provide a maximum of ten individual test wells per slide and stored at –20C until required. For smaller numbers, the slides may be replaced with fixed antigen-infected cells cultured on individual glass cover slips (AFBI, 2012). IFAT is primarily used as a screening method for avian nephritis virus as well as chicken astrovirus suspect samples (Todd *et al*., 2009).

2.4.1.1 HISTORY AND CLASSIFICATION

Astroviruses had primarily been depicted in children faecal testing of diarrhoea (Madeley and Cosgrove, 1975) because of the distinguishing pin-up look as a result of the capsid spike extensions which conferred the name derived from astron (Greek for star). Astroviruses currently are next as important cause of gastroenteropathies to rotaviruses in infantile offspring. Subsequently, astroviruses have been identified in diversity of terrestrial, aquatic and wild livestock, although most of these identifications await comprehensive classification (Bosch *et al*., 2012). Despite the ever-present nature of astroviruses in immature livestock, they are hardly a source of fatalities except in avian species.

Historically, astroviruses were first isolated from various birds, with the initial account of infection depicted in young ducks (Smyth, 2017), nevertheless, this virus identified by electron microscopy as an astrovirus during the1980s (Gough *et al*., 1984) is described as astrovirus serotype 1 (DAstV-1) although it was initially termed duck hepatitis virus 2 (Gough *et al*., 1985). Subsequently, another genetically and antigenically distinct astrovirus of ducks, DAstV-2, was identified as a causal agent of duckling hepatitis (Todd *et al*., 2009). Turkey astrovirus serotype 1 primarily was identified within the United Kingdom in turkeys in 1980 (McNulty *et al*., 1980) while a serotype 2 was detected two decades after (Koci *et al*., 2000).

Efforts at an integrated taxonomy within the *Astroviridae* family have proved difficult because of the high extent of genetic variety and numerous hosts of astroviruses (Bosch *et al.,* 2012). Although Astrovirus was acknowledged as a genus in the *Astroviridae* family in 1995, various modifications had been effected afterwards. Subsequently, taxonomy based only on species of origin grouped astroviruses as

Avastrovirus and Mamastrovirus (Krishnan, 2014). The introduction of molecular approaches for classification however showed the inadequacies of earlier taxonomy methods by the demonstration of assortments of different viruses in a host as well as genetic similarities of identified viruses from various animal sources (Smyth, 2017). Therefore, recent taxonomy scheme suggested categorization centered on nucleotide sequence of the capsid protein gene, proposing that strains with more than 75% similarity in the capsid protein genes ought to be within identical astrovirus group (Bosch *et al.,* 2012). Also, it has been suggested to describe variations in identified astrovirus group when a variant has less than 93% nucleotide homology to archetype strain in a group with capsid gene analysis (Bosch *et al.,* 2012).

2.4.1.2 MAMASTROVIRUS

Mamastrovirus group comprise astroviruses detected from humans as well as terrestrial and aquatic mammals, including animals both domestic and wild that include cattle, sheep, dogs, mink, mice, pigs, camels, deer, cats, bats, whale, dolphins and sea lions (Kemenesi *et al.,* 2014). Nineteen Mamastrovirus groups broadly clustered as GI and GII are currently identified by the ICTV (Woo *et al.,* 2015; Karlsson *et al.,* 2015).

2.4.1.3 AVASTROVIRUS

Viruses detected in several domesticated and wild terrestrial and aquatic birds such as goose, chicken, guinea fowl, dove, turkey, pigeon, penguin, heron and duck have gained recognition within the Avastrovirus group(Koo *et al.,* 2013; Devaney *et al.,* 2016). The astroviruses within initially identified species in the Avastrovirus group include turkey astrovirus type 1 (Avastrovirus 1); avian nephritis virus -1 and -2 (Avastrovirus 2) as well as turkey astrovirus type 2 and duck astrovirus (Avastrovirus 3). However, unclassified astroviruses in avian species, particularly detected in undomesticated terrestrial and aquatic birds are numerous (Bosch *et al.,* 2012).

1. *Avastrovirus* 1

This group comprises two turkey-origin astroviruses; turkey astrovirus serotype 1(McNulty *et al*., 1980) which was identified in 1980 and turkey astrovirus serotype 2 reported in 2000 (Koci *et al*., 2000).

2. *Avastrovirus* 2

Chickens are infected by two astroviruses related to growth retardations, kidney lesions and enteritis in chicks. Primarily detected astrovirus, avian nephritis virus (Yamaguchi *et al*., 1979) in chicks without clinical illness, was originally considered to be a picornavirus but later recognized (Imada *et al*., 2000) to be an astrovirus. The second astrovirus called chicken astrovirus was identified in chickens by 2004 through molecular technique and is a separate species from avian nephritis virus (Baxendale and Metbatsion 2004). Prior to the identification of this second astrovirus, the virus was thought to be an enterovirus because of the resemblance with this genus within *Picornaviridae* family (Baxendale and Metbatsion 2004). Currently, investigations have related chicken astrovirus to hatchery disease and infections in chicks (Todd *et al*., 2009; Devaney *et al*., 2016).

3. *Avastrovirus* 3

The *Avastrovirus* 3 include two duck-origin astroviruses. Duck astrovirus serotype 1 initially was identified to be duck hepatitis virus 2 around the 1980s (Gough *et al*., 1984; 1985). A second astrovirus of ducks, formerly described as duck hepatitis virus 3 is now known as duck astrovirus serotype 2 (Todd *et al*., 2009).

Astroviruses are reported to have a widespread occurrence in young poultry with enteric disease (Devaney *et al*., 2016; Smyth, 2017). Studies have severally shown high prevalence in turkeys and chickens of astroviruses (Koo *et al*., 2013; Kang *et al*., 2018), recurrently related to a variety of enteric viral agents (Todd *et al*., 2009). These infections characteristically happen primarily 28 days of hatch (Reynolds and Schultz-Cherry, 2008). While birds are constantly examined for enteric viral infections on or after hatch until slaughter, the samples primarily are astrovirus positive, with or without other microbes (Reynolds and Schultz-Cherry, 2008).

2.4.1.4 CHICKEN ASTROVIRUS

Chicken astrovirus (CAstV) is an emerging virus identified as a member of avian astroviruses (Baxendale and Mebtsion, 2004). This virus is linked in chickens to runting-stunting syndrome typified by loss of financial viability as a consequence of diminished competence of feed adaption, poor productivity ,deaths (Baxendale and Mebtsion, 2004),

as well as “white chicks” disease related to pale featherings, increased frailty and death of embryos and chicks (Nunez *et al*., 2016). Studies based on the evaluation of hyper variable areas of the open reading frame 2 revealed there is a wide assortment in chicken astrovirus strains (Smyth, 2017) previously known as enterovirus-like viruses in chickens and categorized into two serogroups with slight levels of cross reactivity (McNeilly *et al*., 1994). The identification of low (38-40%) amino acid characteristics within these hyper variable areas further sustained the classification of these two CAstV groupings as A and B. The first (A) group CAstVs comprised three subgroups (Ai-iii), with range of similarity between 77-82%, while B group consist of Bi and Bii, with similarity range between 84-85% (Smyth *et al*., 2012). There are few instances of CAstV detection in other birds such as ducks and pigeons as exemplified by its intermittent findings in them (Awe *et al*., 2015)

2.4.1.5 AVIAN NEPHRITIS VIRUS

Avian nephritis virus (ANV) has variously been detected from chickens with reduced viability as well as intestinal nephritis (Imada *et al*., 2000). The primary ANV serotype (ANV-1) detected from apparently healthy chick was initially regarded as a picornavirus and afterward categorized within *Astroviridae* (Yamaguchi *et al*., 1979; Imada *et al*., 2000). Later, another serotype was detected in underdeveloped chicks as ANV-2 (Takase *et al*., 1989). In addition, periodic findings in turkeys and ducks of ANV 1 and 2 serotypes (Domanska-Blicharz *et al*., 2017) have been reported. There also was further detection in movement disorders and RSS in turkeys and chickens of a third ANV type termed ANV-3 by means of partial nucleotide sequence of the ORF1a fragment (De Wit *et al*., 2011). There is indication of interspecies spread of ANV evidenced by the high similarity of detected ANV in pigeons in Asia with ANV-2 of chickens by the use of phylogenetic analysis (Zhao *et al*., 2011).

2.4.1.6 TURKEY ASTROVIRUS

Although astroviruses have been identified in cases of poor growth and enteropathies in turkeys, these viruses were primarily revealed in spiking mortality disease as a cause of enteropathies and deaths in poults (McNulty *et al*., 1980; Domanska-Blicharz *et al*., 2017). Turkey astrovirus has been categorized into turkey astrovirus type 1 (TAstV-

1) originally discovered in the United Kingdom, with infrequent identification in other birds such as ducks and chickens (McNulty *et al*., 1980) as well as a type 2 TAstV identified by 1996 (Koci *et al*., 2000), principally linked to spiking mortality disease (Pantin-Jackwood *et al*., 2008). Furthermore, within the turkey astrovirus pedigree, type 2 turkey astrovirus has been reported to be greatly diverse and majorly distributed worldwide (Pantin-Jackwood *et al*., 2008).

2.4.2 PROPERTIES OF THE VIRAL PARTICLES

Viral particle of astrovirus is not enclosed, of about 28–33 nm span with icosahedron proportion (Reynolds and Schultz-Cherry, 2008). The host cell for virus cultivation as well as virus strain may influence astrovirus morphology demonstrated by the smooth appearance of most viral particles in negative-stained sample preparations as against only minute numbers with the characteristic barbed exterior protrusions resembling stars (Matsui and Greenberg, 2001; De Benedicts *et al*., 2011). The precursor capsid protein is initially gathered as a virion but through cleavage develop into viral capsid proteins. The precursor capsid protein is anticipated to be involved in viral RNA packaging and receptor binding at the N and C termini, respectively (Matsui and Greenberg, 2001). The distinct RNA fragment (single –stranded) with positive polarity in the genome, 6.4-7.9 kilobases by magnitude consist of open reading frame 1 to 3(Matsui and Greenberg, 2001). Although the 3' end of the genome is polyadenylated, depending on the strains, untranslated areas of variable length are contained at the two ends (5' and 3'). Lipid solvents, low pH and detergents have low effect on virus infectivity (Schultz-Cherry *et al*., 2001).

2.4.3 REPLICATION OF VIRUS

Astroviruses of human and animal origins are capable of growth in cell cultures. While growth of astroviruses is not sustained by most cell lines, the human cell line (Caco-2) allows growth of human astroviruses exclusive of host-cell adaptation (Cann, 2008). During virus propagation in cell culture, maturation of viral particles is aided by the addition of trypsin. However, trypsin is not required in primary chicken kidney cells for chicken astroviruses growth (Baxendale and Mebatsion, 2004). The capsid protein

portion, C-terminal, is proposed to cause the establishment of cell tropism through binding of cell. The specific cell receptor(s) to which astroviruses bind has not been clearly identified, but preliminary studies have implicated the polio virus receptor (CD155) for one human astrovirus Even though CD155, the polio virus receptor, has been linked to a human astrovirus, precise binding cell receptors of astroviruses are yet to be definitely classified (Cann, 2008). Replication of viral particles occurs in the cytoplasm with assortment within the cytoplasmin crystalline of matured viral particles and subsequent release by lysis of infected cells (Cann, 2008).

Polyproteins composed of non-structural proteins of astroviruses are predetermined by the virus RNA genome that also operates as a messenger RNA for open reading frame 1a and 1b which are copied via frame shifting means (Matsui and Greenberg, 2001), however, there are no strict definitions yet of the open reading frames. The serine protease region predetermines the localization signal and trans-membrane area within the nucleus which may be mostly dependent on species involved. The structure of the viral proteins of the genome is determined at the 3' end by another open reading frame (Cann, 2008).

2.4.4 VIRUS DISTRIBUTION

Infections by astroviruses are in nature commonly widespread with the infection reported to have a wide-ranging occurrence in young poultry with enteric disease and poor growth (Devaney *et al*., 2016; Smyth, 2017). Several studies have demonstrated that a high number of poultry flocks are prone to infections by astroviruses, often in relationship with other enteropathogenic microbes (Smyth, 2017; Kang *et al*., 2018; De laTorre *et al*., 2018). Intermittent detections of astroviruses in normal healthy flocks are also reported (Devaney *et al*., 2016). Infections with astrovirus typically arise preliminarily within initial 28 days of hatch (Smyth, 2017). In addition, poultry flocks constantly screened for enteric viral infections from hatch until slaughter, always contain astroviruses, either alone or with other viruses (Dai *et al*., 2010)

2.4.5 VIRUS DIAGNOSIS

Assessment of astroviruses were initially via electron microscopy utilizing sample preparations from faeces of affected animals or humans, however, morphological variations due to pH alterations as well as the small virus size often cause misidentification of astroviruses as just a round tiny virus resembling an enterovirus (van Regenmortel *et al*., 2000). Accurate diagnosis of astroviruses by means of electron microscopy is usually hindered in cases of multiple infections by different viruses (Schneider and Roossinck, 2001). Nucleic-acid-based detection systems which include assays like the reverse-transcriptase polymerase chain reaction are noted to have substantially improved avian astroviruses testing by increased analytical specificity and sensitivity (Smyth *et al*., 2009). Consequently, this to a great extent will make possible enhanced description of the distribution and consequence of astrovirus infections.

2.4.6 SUSCEPTIBILITY OF ASTROVIRUSES TO PHYSICAL AND CHEMICAL AGENTS

Astroviruses are very stable to the effects of chloroform, detergents, acidic pH, heat, ambient temperatures, quaternary ammonia, alcohols, and lipid solvents inactivation (Schultz-Cherry *et al*., 2001). Methanol (90%), potassium peroxymonosulfate-containing disinfectant, b-propriolactone, and formaldehyde have been revealed to reduce astrovirus contamination. Embryo intestine-derived turkey astroviruses are especially stable and infectivity can be sustained at fridge temperature or up to –70°C for short or prolonged time (Schultz-Cherry *et al*., 2001).

2.4.7 PATHOGENESIS AND EPIDEMIOLOGY

Infections by astrovirus are recognized in chickens, by means of their antibodies identified within poultry birds. However, endeavors to ascertain dynamic infectivity in non poultry are so far not conceded. Although day-old chicks are more predisposed, birds within different ages can be infected (de Wit *et al.,* 2011). Direct and indirect spread of the virus has been indicated via contaminated eggs and environment (Koci, 2005; Domanska-Blicharz *et al.,* 2011).

The sole symptom observed clinically in infection by astrovirus in just hatched birds is diarrhea (usually brief) however; this sign may not be observed in all chicks (Matsui and Greenberg, 2001). Additionally, astrovirus virulence, species of bird as well as investigational settings may affect fatality rates (Smyth *et al.,* 2010). Naturally, clinical pathologies linked to this disease in broilers include in apparent signs to occurrence of malabsorption syndrome and baby chick nephropathies (De Benedictis *et al.,* 2011). Little information is acknowledged about clinical signs in turkeys.

2.4.8 DISEASE MANAGEMENT

There is no reported effective prevention or control of avian Astrovirus infections by specific chemotherapeutics, vaccines or other measures. Sellers *et al*. (2010) however showed that multiple vaccinations of breeders with a recombinant variety of the chicken astrovirus ORF2 protein proffered a little protection to RSS- challenged hatchlings, indicating that this approach deserves further investigation. The difficulty in the riddance of the widespread episodes of astroviruses in commercial poultry is worsened by the resistance to inactivation by most generally accepted disinfectants and environmental stability (Schultz-Cherry *et al*., 2001). It is possible that use of effective disinfectants, increased down-time before restock, and strict biosecurity may possibly decrease the chance of astrovirus infection; nevertheless, this application calls for additional study.

2.5 CHICKEN AND TURKEY PARVOVIRUS

2.5.1 HISTORY AND CLASSIFICATION

Numerous diseases of importance across different groups of animals are caused by parvoviruses (Breslin *et al*., 2000). Parvoviruses are interrelated regardless of their multifarious classification structure and commonly have genetic attributes such as ability to withstand drought conditions and DNA replication by means of mitotic S phase cell transition (Breslin *et al*., 2000). The accessibility of these actively dividing cells grants propensity for parvovirus infection in particular tissues depending on the age of such (Dhama *et al*., 2009). Consequently, severe infections by parvoviruses occur in young animals and foetuses through the placenta (Breslin *et al*., 2000).

Turkey and chicken parvoviruses belong to the family *Parvoviridae*. Comparison of the genomic configuration by sequence analysis showed that they ought to appear as a novel group in vertebrate-subfamily *Parvovirinae* (Breslin *et al*., 2000). The primary turkey and chicken parvoviruses were recognized by transmission electron microscopy in gut samples from broiler chicks affected by stunting disease (Kisary *et al*., 1985) during the early 1980s. Equally, turkey parvoviruses were identified in enteric infection leading to intestinal intranuclear inclusion in turkeys (Trampel *et al*., 1983). Since the first discoveries, parvoviruses were subsequently related to enteric maladies of birds (Dhama *et al*., 2009), which may involve hypoplasia of the cerebella in day-old broilers (Domanska-Blicharz *et al*., 2012), and in fecal samples of wild turkeys (Breslin *et al*., 2000). Furthermore, the determination of whole genome sequences of chicken and turkey parvoviruses has enhanced diagnostic assays development employed in establishing the occurrence of parvoviruses in poultry production (Domanska-Blicharz *et al*., 2011). Parvoviruses are often identified in turkeys and chickens with enteric maladies (Domanska-Blicharz *et al*., 2012). More so, infectivity studies show that turkey and chicken parvovirus stimulate characteristic enteric maladies in predisposed immature poultry (Bloom and Kerr, 2006; Dhama *et al*., 2009). Parvovirus infections occupy an important part in the aetiology of enteric conditions of birds; such as spiking mortality and runting-stunting syndrome even though its precise economic implication has not been determined (Bloom and Kerr, 2006; Dhama *et al*., 2009). Chicken and turkey parvovirus are not of zoonotic importance.

Categorization of parvovirus by phylogenetic analysis utilizing sequences of virus genomes has grown to be the principal method to categorize parvoviruses. This technique has replaced earlier system based on natural configuration of the virus (Tattersall, 2006; Day and Zsak, 2010). Genome sequence analysis clearly specified the similarity of chicken and turkey parvoviruses and their notable difference from others affiliated to the subfamily or family (Tijssen *et al*., 2012).

2.5.2 VIRUS CHARACTERISTICS

Parvovirus viral particles are non-enveloped of icosahedron proportion and a width of 25 nm. Majority (about 90%) of the 60 molecules of protein that make up the

parvovirus capsid formed by singular mRNA substitution-merging are the viral protein 2 partly covered by the bigger viral protein 1 gene (Tattersall, 2006). Viral protein 3 could also be produced in few instances by peptide splitting from the viral protein 2 termini (Tattersall, 2006; Day and Zsak, 2010). The capacity of parvovirus to remain stable in the environment, elicit antigenicity and bind to receptors depends on the extensive circling of the inner heterologous β-tub pattern of the capsid proteins (Day and Zsak, 2010). In general, sanitization of parvovirus-contaminated premises is usually difficult due to the resistance of this virus to biological (high pH and heat) and chemical sanitization means.

A linear DNA that is of a single strand is contained in the genome of about 6 kb in size that ends in little palindromic sequences that are capable of forming double hairpin telomeres at either ends of the genome (Tattersall, 2006). The viral structural protein of the capsid is predetermined at the open reading frame within the 5’ end as well as the non-structural viral protein essentially involved in DNA production and duplication predetermined within the 3' end of identical strand on parvovirus DNA genome (Tattersall, 2006).

Supplementary analyses on the chicken parvovirus genome configuration revealed that the viral particles include a solitary genome that act like an *in vitro* model for production of subsequent strand accompanied by suitable precursors and DNA polymerase (Kisary *et al*., 1985) with migration of the transformed linear DNA of about 5.2 kb in agarose gels. The structures of the viral particles collectively with their biochemical properties were typical of the members of the *Parvovirus* family (Muzyczka and Berns, 2001; Day and Zsak, 2010).

Generally, the genome configuration of turkey and chicken parvoviruses is analogous to others, through envisaged open reading frames (ORF) that are majorly two. The viral nonstructural protein coded by 5' ORF is of significance to the virus pathogenesis and duplication commencement (Muzyczka and Berns, 2001) while the parvovirus capsid proteins coded by the major 3' ORF contain the viral particle essential for counteracting antibody stimulation in the course of a disease (Muzyczka and Berns, 2001).

2.5.3 VIRUS REPLICATION

Turkey and chicken parvoviruses have replication capability without helper viruses (Kisary *et al*., 1985). Immunoassay and electron microscopy studies demonstrated the small intestine as location of the principal cells meant for *in vivo* replication (Kisary *et al*., 1985; Zsak *et al*., 2009). There is limited knowledge about the replication mechanisms of turkey and chicken parvoviruses in infected cells as a result of cultivation incapability in avian embryos or cell cultures (Zsak *et al*., 2009).

Generally, the pathogenesis of parvovirus infections is apparently influenced by the binding preference of precise viral particles for receptors even though the majority of parvoviruses of animal origin do not adequately possess tissue- receptor specificity to elucidate virus tropism (Cotmore and Tattersall, 2006). Within the cell cytoplasm, there is early, late and recycling endosomal passage of viral particles which is yet clearly defined (Soares *et al*., 1999). On the other hand, the alteration of the membrane of the endosomes to make possible the release of viral capsid may possibly be due to the enzymatic action of phospholipase A2 exclusively contained in the N terminal of viral protein 1 hidden within recently made viral particle (Soares *et al*., 1999; Tattersall, 2006).

The parvovirus DNA is copied to form a transitional second strand by the cellular enzyme, DNA polymerase which is then utilised in virus replication via virus mRNA transcription. The unconventional linkages of varieties of mRNA produce the minor and major proteins (Bloom and Kerr, 2006). Viral structural protein assembly is regulated at the 5' end of the genome while the 3'end predetermines the nonstructural viral protein responsible for the nonstructural viral protein attachment to 5' end, DNA enclosure and duplication, as well as enzyme-regulation of DNA arrest mediate (Cotmore and Tattersall, 2006). Although the replication method of the genome is intricate and not totally implicit, it is expressed as an undulating hairpin; with the DNA with negative polarity having a 3' end providing basic self coverage for the commencement of transitional second strand required for replication of DNA (Cotmore and Tattersall, 2006).

2.5.4 PATHOGENESIS AND EPIZOOTIOLOGY

Recent studies showed turkey and chicken parvoviruses to be widespread in poultry in America and numerous countries of Europe (Day and Zsak, 2010; Koo *et al*.,

2013). The surveys that mainly utilised specific diagnostic PCR assays to detect turkey and chicken parvoviruses in faecal or intestinal contents from commercial turkey and chicken flocks obtained from diverse areas showed parvovirus infection in poultry flocks at majority of the key poultry-producing centers, especially in birds as early as four days after hatch. Furthermore, these reports revealed that nearly all of the positive flocks had genetically similar circulating strains of the virus.

Although turkey and chicken parvovirus infections have been observed in commercial turkeys and chickens as the natural hosts, fast-growing chickens are mostly at risk of viral infections (Zsak *et al*., 2009). In addition, the inclination to parvovirus-related enteric infections in poultry is robustly age associated (Domanska-Blicharz *et al*., 2012) with occurrence of majority of recurrent infections within the first few weeks of life. However, older birds produce virus-specific serum antibodies without overt signs of infection (Day and Zsak, 2010).

The shedding of high quantities of the virus in the faeces of infected birds usually results in efficient and swift disease diffusion (Day and Zsak, 2010). In addition, the stability of parvovirus in surroundings allows easy identification in wastes, thus presenting added contamination resource for chicks restock (Day and Zsak, 2010). Also, feral birds may be considered as possible avian parvovirus reservoir (Saif, 2013). The major clinical signs related to naturally occurring contagious enteric infectious condition of birds related to turkey as well as chicken parvovirus infections can be described as PEMS and RSS of turkey poults and chicks respectively (Day and Zsak, 2010; Saif, 2013).

2.5.5 DIAGNOSIS

The initial focal method of identification of parvovirus infections in turkey and chicken flocks was the electron microscopy (Day and Zsak, 2010). Serologic assays (such as indirect immunofluorescence and enzyme-linked immunoassays) to identify viral antigens or antibodies are commonly used investigative tools to monitor parvovirus infections in poultry flocks and to evaluate the protection profile of birds (parent and their progeny) (Dey, 2003; Strother and Zsak, 2009).

Diagnostic PCR tests targeting the nonstructural genes of turkey and chicken parvoviruses have been developed as a result of high level of similarity of these genes

specified by comparative genome sequence analysis (Tarasiuk *et al*., 2012). These diagnostic tests verified to be highly sensitive and specific are increasingly utilised in poultry parvoviruses detection (Domanska-Blicharz *et al*., 2012).

2.5.6 PREVENTION AND CONTROL

The extensive prevalence of parvovirus in birds implies the low possibility to maintain disease-free flocks (Zsak *et al*., 2013). Strict biosecurity procedures are essential to prevent virus accumulation in the environment and manage parvovirus transmission via faeces, waste and apparatus (Dhama *et al*., 2009). Parvovirus is remarkably stable and may continue to exist for some period within the surroundings (Tattersall, 2006). Several reuse of litter coupled with a short down-time between flock restocks, may enhance infection of the birds with possible consequence of disease devastation in contrast to flock where clean litters is utilised often (Tattersall, 2006). Commercial vaccines are currently unavailable to prevent parvovirus-induced enteric diseases in poultry and this is apparently challenged by the complexity to grow the virus (Day and Zsak, 2010). However, studies have revealed that maternal antibodies to poultry parvoviruses is able to decrease the disease fatality signifying that parent stock vaccination may possibly protect at risk young birds after hatch (Tarasiuk *et al*., 2012).

2.6 AVIAN ROTAVIRUS

Rotaviruses are acknowledged as the principal aetiologic cause of virus-related enteropathies and diarrhoea in man (primarily children) as well as in the infant of several mammalian groups (Franco and Greenberg, 2009). Avian rotavirus was primarily recognized in the course of analyzing the gut content samples for enteritis in young turkeys in America and Europe (McNulty *et al*. 1980) and had since been detected in diverse domestic and wild species of birds (McNulty, 2003; Dhama *et al*. 2009). Although rotavirus infections have an effect on young and adult birds, high infection vulnerability and mortality are majorly noticed in immature birds (1-2 weeks) are most susceptible with high mortality (Dhama *et al.,* 2009). Avian rotavirus infection is often linked with occurrence of diarrhoea and wide-ranging flock depression, and is frequently found involved with malabsorption syndromes in poultry (Dey, 2003). Financial impact in

related enteric disease to rotavirus in poultry production is unclear, but nonspecific enteric syndrome with an apparent viral aetiology is an ongoing industry burden (Dey, 2003; Dhama *et al*., 2009).

Some avian rotaviruses have been shown not to be strictly species- specific evidenced by the capability of rotaviruses from pheasant and turkey to transmit disease to specific-pathogen-free birds (Yason and Schat, 1986). Likewise, group A rotaviruses of mammalian sources have also been identified in birds with diarrhoea (Tarasiuk *et al*., 2012). Despite field as well as experimental indication of interspecies spread of rotavirus involving mammals and poultry, it is assumed to be of infrequent occurrence (Martella *et al*., 2010). Thus, avian rotaviruses are not zoonotic and have no recognized public health importance (Martella *et al*., 2010).

2.6.1 VIRUS, CLASSIFICATION AND GENOME ORGANIZATION

The avian rotaviruses belong to the *Reoviridae* family, typically characterised by viral particles containing linear double-stranded RNA with10 to 12 segments (King *et al*., 2012). *Reoviridae* is comprised of fifteen genera grouped as two known subunits according to existence and lack of structural "shoot ups" positioned by the side of external capsid vertices. Within the non-shoot up subunit, *Sedoreovirinae*, is *Rotavirus* genus, while shoot-up genera include the subfamily *Spinareovirinae* (King *et al*., 2012). Although *Reoviridae* cause infections of animals and non-animals, rotaviruses are capable of infecting animals solitarily through means of contaminated feed and water (Desselberger, 2000). Rotaviruses are made up of eleven genome segments with double RNA strands having conserved 5' and 3' ends. Every one of the genome fragment encodes solely a virus protein, but two virus proteins are coded for by the eleventh segment (Desselberger, 2000; Jiang *et al*., 2008). Observation through electron microscopy revealed three-coated capsid enclosed genome looking like a typical wheel from where the virus name was coined (Desselberger, 2000). The assembly of rapidly enveloped particles in the endoplasmic reticulum is involved in rotaviruses replication and genetic reassortants having an assortment of genome segments from each parent virus possibly could arise as consequence of co- infections with two different strains of rotavirus in susceptible cells (Desselberger, 2000; King *et al*., 2012).

Mature viral particles of rotavirus are generally about 1000 Å in diameter, non enveloped and triple-layered icosahedrons with the VP4 cell accessory protein spikes (Jiang *et al*., 2008). However, the three layered viral particle without the projecting spike is about 750Å in diameter (King *et al*., 2012). This presents an even exterior of the external protein covering that differentiates rotavirus from other *Reoviridae* by the use of electron microscopy (King *et al*., 2012).

2.6.2 VIRUS REPLICATION

Viroplasms are typical electron-dense cytoplasmic inclusion bodies displayed by rotavirus-infected cells and are basically where duplication and primary covering of the virus genomic fragments take place. The affinity of specific protein structures intended for recently formed positive-sense RNA possibly regulates these inclusion bodies formation (Patton *et al*., 2006). The newly translated positive-sense RNA genome segment precursors are connected involved to the viral central configuration by the self-assembly of structural proteins of the viral particles that occur in the viroplasms (Jiang *et al*., 2008). The double stranded RNA genome replication occurs entirely in the newly formed viral hubs which consist of copies of virus protein 1 enzyme RNA-polymerase, structural protein (VP2) and viral methyl-transferase and guanylyltransferase (VP3). Thus, the double strands of RNA genome fragments are in no way exposed to the cell core (Jiang *et al*., 2008). In addition, the segment transcription of each genome devoid of absolute uncoating of the double stranded RNA is allowed by the connection of the intact viral core with the rotavirus transcriptase complex (Patton *et al*., 2006; Jiang *et al*., 2008). The double-layered rotaviral particles emerge after acquisition of structural protein VP6 from the viroplasm appearing to develop through the VP7 endowed membrane patches transiently into the endoplasmic reticulum (Pesavento *et al*., 2006). The external capsid proteins VP4 and VP7 are then added to the non-enveloped triple layered rotaviral particles. This final course of maturation is however inadequately understood (Pesavento *et al*., 2006).

2.6.3 PATHOGENESIS AND EPIZOOTIOLOGY

Studies utilizing traditional and molecular approaches have extensively detected avian rotaviruses in various poultry species in the Americas, Europe and Asia (Jones *et al*., 1979; Jiang *et al*., 2008). Rotavirus infection naturally occurring in birds and related signs of enteric disease usually occur in young (less than 42 days old) birds (Barnes and Guy, 2003). However, experimental rotavirus infection of turkeys and chickens indicate higher susceptibility of older (8-17 weeks) birds than younger ones (Yason et *al*., 1987). Similarly, studies have shown occurrence of rotavirus-related diarrhoea in 32-92 weeks old commercial layer birds (Jones *et al*., 1979). Rotaviruses are usually voided in high quantities in faeces of birds (Yason *et al*., 1987), as such; cloacal swabs can be easily used for molecular detection of the virus (Spackman *et al*., 2010).

Similar to mammalian rotaviruses, environmental contamination of avian rotaviruses in faeces is possibly persistent enhancing occurrence of horizontal transmission between birds via direct and indirect contacts (Spackman *et al*., 2010) as well as the speculations of egg contamination which possibly could happen within or on the shell (Pantin-Jackwood *et al*., 2008).

2.6.4 DIAGNOSIS, PREVENTION AND CONTROL

Laboratory diagnosis of avian rotavirus is typically through the identification of the virus in faeces by electron microscopy. This detection procedure is comparatively sensitive and able to identify all serogroups of rotaviruses; however, it is costly and cumbersome (Dhama *et al*., 2009). Alternatively, detection of rotavirus RNA in faeces and identification of migration pattern of the eleven genome segments on polyacrylamide gels is not only as sensitive as electron microscopy but provide momentary knowledge on the serogroups available making it an expedient way to differentiate diverse virus isolates (Dhama *et al*., 2009).

Rotavirus diagnoses through in vivo propagation of cells appear more valuable for A- rotaviruses because of the established difficulty of cell culture isolation of other rotavirus serogroups (Dhama *et al*., 2009). Consequently, virus isolation in cell-culture is hardly suggested as an analytical method because non-group A-rotaviruses make up a greater part of infection causes within turkeys and chicken (Jindal *et al*., 2012).

Similarly, serologic diagnosis is complex and also infrequently suggested. Moreover, elevated occurrence of antibodies (Dhama *et al*., 2015) makes results difficult to interpret. Furthermore, the inability of a number of these viruses to adapt to cell-culture has led to deficits in availability of antigen sets (Dhama *et al*., 2015). While usage of immunoassays are valuable in serologic testing to establish and examine the condition of SPF flocks (McNulty, 2003), individual RT-PCR tests aimed at definite genomic fragments have enabled detailed molecular characterization of avian rotavirus (Domanska-Blicharz *et al*., 2012) and these assays have been employed to differentiate avian rotavirus groups in field samples. Furthermore, as more genomes of avian rotaviruses are wholly characterised and interpreted, accessibility to new molecular diagnostic tests becomes more feasible (Dey, 2003).

The widespread occurrence of rotavirus infections in chicken and turkey shows routine efforts to maintain disease-free flock is not practical (Dhama *et al*., 2015). No precise treatment or means of control exists presently. Moreover, vaccine development may be a problem considering the complexity of *in vivo* propagation in cells of non-group A rotaviruses (Dey, 2003).

2.7. AVIAN REOVIRUS

Every commercial poultry production in all probability may be affected by avian reovirus (Kort *et al*., 2013). Avian reovirus covers a wide detection rate in chickens impinged on by an assortment of disease manifestations such as runting-stunting syndrome, viral tenosynovitis or arthritis, respiratory and enteric diseases and osteoporosis (Liu *et al*., 2004). However, in the case of runting-stunting syndrome, there is no existing substantiation of this virus as a key source of the disease. In addition, avian reoviruses have been found frequently in clinically unaffected chickens. The severity and disease characteristics in poultry depend upon virus virulence, host age, and route of exposure (Kort *et al*., 2013).

Worldwide, avian reovirus is of varying significance in different poultry production which may be due to rearing management especially of meat type birds, ease of virus detection and virulence of strains present in particular production (Liu *et al*., 2004).

2.7.1 VIRUS PROPERTIES

Avian reoviruses belong to the genus Orthoreoviruses. The entire virus particles are non-enveloped, about 85 nm in length as well as sphere-shaped containing outer protein shell of icosahedron proportion with numerous layers. There are linear double strands of ten RNA fragments of genome consists of linear double stranded RNA divided into 10 segments, distinguishable as small, medium and large moieties (Schnitzer, 1985). The S1 fragment contains a principal open reading frame (ORF) that predetermines the small outer shell (Sigma C) protein (Kant *et al*., 2003). Studies with reassortant reovirus of birds illustrated the pathogenesis and replication involvement of the virus by the S1 segment (Kant *et al*., 2003; Huang *et al*., 2011; Kort *et al*., 2013).

A virtually globular icosahedron with μ1C and σ3 protein multi parts is outlined by the surface capsid (Shih *et al*., 2004). Twin unwavering sub viral units are structured besides the integral viral particles; the infectious sub viral unit missing only the external protein shell and the core particle which lacks the middle and external protein shells (Huang *et al*., 2011). The μ1protein with outward spike appearance on the surface of the capsid allows the viral particles attach to cells of the host, and this protein even in the absence of the surface capsid stay connected as extensive cellular fibers at the tips of the sub viral infectious particle. Each of the 10 fragments of orthoreovirus genome predetermines a distinct protein with the exception of a cleaved fragment that appears double. Furthermore, the electropherotype outlines determined by size of each fragment distinguished by gel electrophoresis have been utilised to categorize virus isolates from birds and mammals (Huang *et al*., 2011). Orthoreovirus infectivity is less inactivated by lipid solvents compared to ethanol, formalin and phenols (Shih *et al*., 2004).

2.7.2 REPLICATION OF ORTHOREOVIRUSES

A diversity of σ 1orthoreovirus proteins mediate virus attachment to target cells via receptors that depend on virus serotype such as sialylated glycoprotein as well as the junction-adhesion molecule A that does not (Salsman *et al*., 2005). Entry of virus or sub viral infectious particles into cytoplasm of predisposed cells allows their breakdown into a nucleus of particles that facilitate the utilization of the negative strand of the genome

fragment aided by polymerase enzymes to produce mRNA required for virus RNA transcription (Shih *et al*., 2004). Although replication system of the RNA genome appears intricate and not yet totally comprehensible, a mass of newly produced mRNA are favorably translated to viral particles through assemblage of their structured proteins while synthesis of protein by the infected cell decline rapidly. Thereafter, inclusion bodies within the cell cytoplasm are created with variations in morphological configurations that ultimately get extruded when there are lyses of affected cells (Huang *et al*., 2011).

2.7.3 PATHOLOGIC PROCESS AND EPIDEMIOLOGY OF ORTHOREOVIRUSES

Experimental system that utilises a model that involves neonatal infection of mice has enjoyed widespread recognition in the analysis of orthoreovirus pathogenesis (Kort *et al*., 2013). Spread of virus is majorly through the ingestion of contaminated feed and water which lead to a general infection of the host system; however, nondescript inflammatory infiltrations and focal necrotic lesions frequently appear in a number of organs. More so, haemorrhage, inflammation and necrotic lesions within joints and tendon of affected birds are commonly observed (Kort *et al*., 2013). In addition, organs such as spleen, liver and kidney have been shown to have necrotic lesions mostly in ducks and rarely in chickens affected either solely by this virus or with other infectious microbial causes (Zhang *et al*., 2006; Kort *et al*., 2013).

2.7.4 DIAGNOSIS

Avian orthoreovirus infections are routinely identified typically by serological techniques that employ the virus antigen, which include enzyme linked immunosorbent test as well as immunofluorescence test (Jones, 2000). Likewise, utilization of cell culture systems to separate the virus typically portrayed by formation of syncytium as well as vacuoles in infected culture media and further characterised using neutralization test (Shih *et al*., 2004; Salsman *et al*., 2005). Molecular approaches to identification of avian orthoreovirus have been described using restriction enzyme fragment length polymorphism, dot-blot hybridization as well as reverse transcriptase polymerase chain reaction (Roussan *et al*., 2012)

2.8. FOWL ADENOVIRUS

2.8.1 HISTORY AND CLASSIFICATION OF ADENOVIRUSES

In the course of studies on naturally deteriorating adenoids from humans; a novel pathogen, adenovirus was identified and later associated with infectious hepatitis of canine as an etiological agent (Berk, 2006). Since then, various birds and mammals as well as humans have had particular adenoviruses detected in them and possibly indicate the specificity of these viruses maintained in particular host species (Berk, 2006). While majority of adenoviruses generally induce inapparent infections, fatal maladies are linked with these viruses found in avian and canines (Liu *et al*., 2004).

The family *Adenoviridae* currently involves discrete genera based on serology that include genus *Mastadenovirus*, involving only mammal-infecting viruses; genus *Aviadenovirus*, involving only avian-infecting viruses; a third genus *Atadenovirus* with an extensive host range of domestic and wild animals susceptibility to infection as well as a fourth genus *Siadenovirus*, involving adenoviruses 1 and 3 of frogs and turkeys, respectively and some emerging viruses in wild animals (Benko *et al*., 2000; Berk, 2006). Fish (for instance white sturgeon) adenovirus is an anticipated fifth genus (Benko *et al*., 2002). Despite the morphological comparability of adenoviruses (Plate 2. 1) the association of the genome (Plate 2.2) is at variance within the genera containing diverse viruses (Berk, 2006).

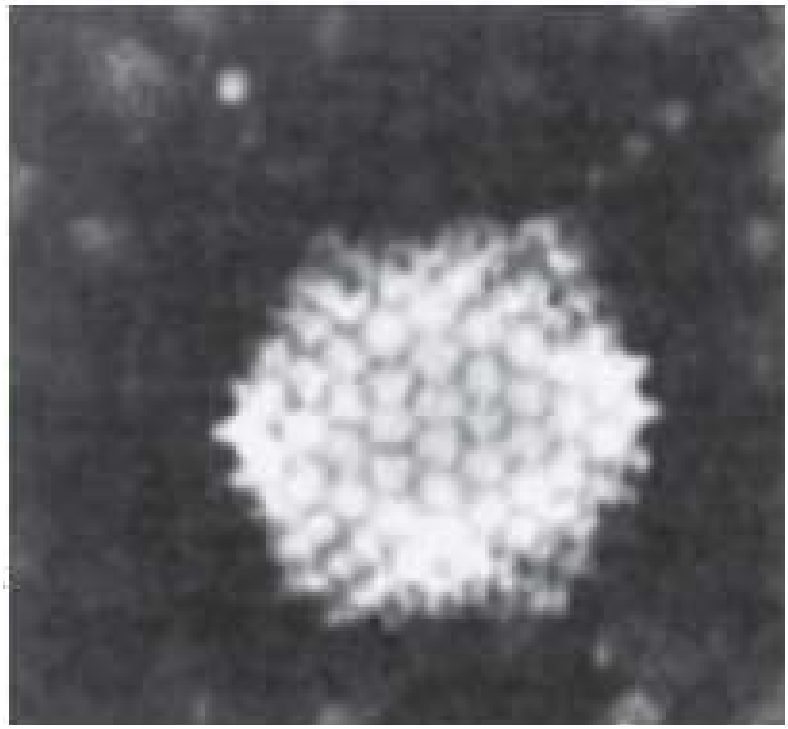


Plate 2.1: Transmission electron (negative contrast) micrograph of adenovirus-2 particle (Benko *et al*., 2000)

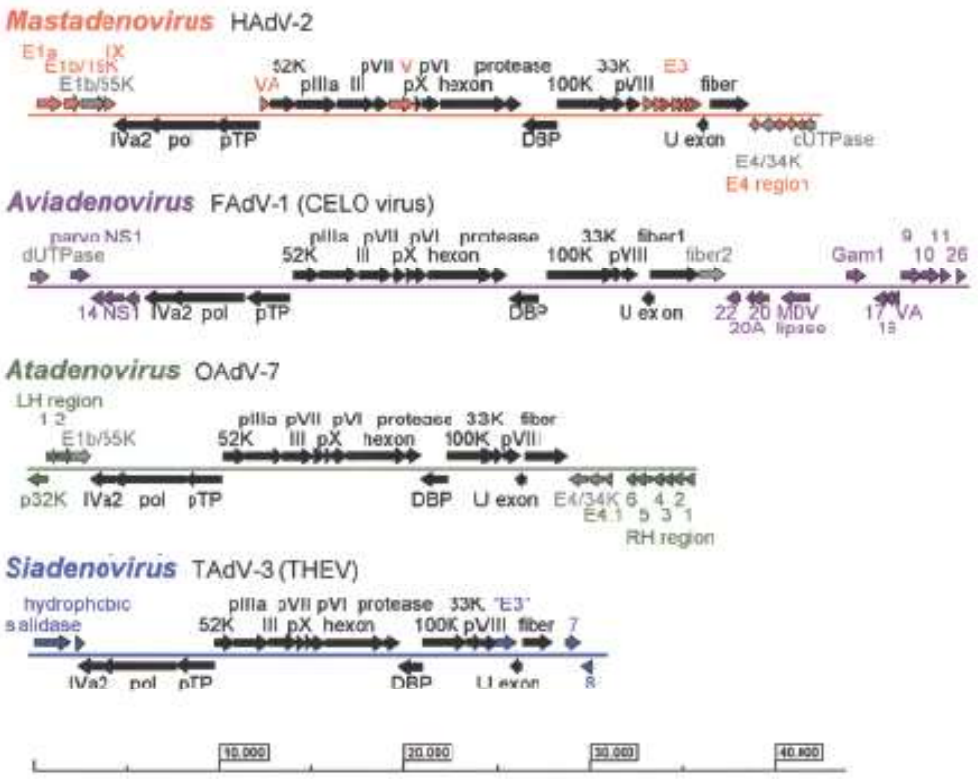


Plate 2.2: The diverse categorizations of adenovirus genomes in the 4 established genera graphically expressed. The arrows with black colour show genus-conserved genes, arrows with grey colour illustrate non specific genes while the bright coloured arrows illustrate genes explicit for each genus. Legend: Fowl adenovirus-1 (FAdV-2), Human adenovirus - 2 (HAdV-2), Ovine adenovirus-7 (OAdV-7), turkey adenovirus-3(TAdV-3) (Courtesy: Fauquet *et al*., 2005)

Description of adenoviruses is by means of the host type as well as a number series (for instance, turkey adenovirus 3). The varieties of host, interrelatedness of adenovirus genome as well as their distinguishing growth patterns in cell culture have all been utilised in virus strain classification in addition to initially used serological characterization (Liu *et al*., 2004; Dimmock *et al*., 2016). The reconfiguration of adenoviruses utilizing their molecular distinctiveness further enhanced the comprehension of their immunologic associations. In particular, determination of antigenicity linked with the epitope-containing structural element (hexon) of the virus capsid that was primarily employed in the characterization of the virus class (Liu *et al*., 2004; Schachner *et al*., 2016).

2.8.2 PROPERTIES OF ADENOVIRUSES

The entire adenovirus particles with diameter of about 90 nm appear hexagonal, of icosahedron proportion and non enveloped (Dimmock *et al*., 2016). The hexons of about 240 featured in the icosahedron surface are separated into a concave-centered false hexagonal base as well as three towers incorporated-top with triangular outline; all bearing five-sided fiber projections (Dimmock *et al*., 2016).

The adenovirus genome is comprised of a solitary linear particle of DNA with double strands, a dimension of about45 kbp, having terminal replicates that are overturned (Berk, 2006). Around forty transcribed proteins as a result of multifaceted merging of RNA are predetermined by genome of adenovirus, with only a minor part of these proteins. An enzyme essential for precursor proteins development predetermined by the virus, cysteine protease, is incorporated with the structural proteins linked by means of the pentons and their strands as well as hexons to the virus particle hub that form around thirty three percent of the transcribed proteins (Dimmock *et al*., 2016).

Even though these viruses can be simply made passive by regular disinfecting agents, they still enjoy comparative ecological stability. The host range of majority of these viruses appear narrow, nonetheless, adenovirus-1 which mainly affects canines in addition was associated with outbreaks in wildlife (Liu *et al*., 2004; Dimmock *et al*., 2016). Generally, varying degrees of fatalities connected to gastroenteric or respiratory maladies are caused by the various adenoviruses (Dimmock *et al*., 2016).

2.8.3 REPLICATION OF VIRUS

The replication of adenoviruses in the nucleus is made possible by the wide-ranging immune response modulation of the host whereby the protuberances of the penton strands attach the viruses to receptors of the host cells and subsequently engulfed (Cann, 2008; Schachner *et al*., 2016). This is followed by the detachment of the external protein covering (capsid) to expose the entry of central part of the virus genome into the nucleus for the transcription, replication and assembly of mRNA, virus DNA and viral particles, respectively (Kasamatsu and Nakanishi, 1998). Thus, there is crystalline collection of viral particles in the nucleus leading to irregular appearance of the nuclei due to the rigorous compression as well as margin-placement of chromatin of cells of the host. Eventually, there are lyses of these cells to release the viral particles (Berk, 2006).

2.8.4 AVIADENOVIRUS

The serologically unique aviadenovirus genus is linked with a number of significant infections in avian species which they solitarily infect. The pathogenicity of a large number of viruses in this genus is yet clearly described except the viruses responsible for quail bronchitis and hydro pericardium syndrome (Balamurugan and Kataria, 2004). Aviadenoviruses, formerly categorized as Subgroup I adenovirus of avian, comprise adenoviruses of fowl (1–11), pigeon, turkey (1-2) and duck (2). Investigational studies are deficient and unable to reproduce uncomplicated disease scenario despite the diverse associated disease syndromes with aviadenovirus typified by erosions of gizzards, drop in egg-laying or pace of growth, inclusion-body hepatitis, tenosynovites, and respiratory ailments observed in variety of avian species (Balamurugan and Kataria, 2004).

2.8.5 PATHOGENESIS AND EPIZOOTIOLOGY

Conventional adenoviruses are spread both vertically and horizontally, mainly through ingestion of contaminated feed and water. These infectious viruses may replicate within uterus of hens and transmitted either in or on the egg and also in droppings contaminated by secretions from the oviduct. Because many healthy birds are infected

with conventional adenoviruses, it is difficult to determine the importance of adenoviruses as pathogens (Benko *et al*., 2000). Strong evidence exists that some strains can cause inclusion body hepatitis disease and pancreatitis in birds. There are also indications that adenoviruses are associated with the hydropericardium syndrome or Angarra disease (Balamurugan and Kataria, 2004).

2.8.6 DIAGNOSIS

Analyses involving histology and serology form the major identification basis for adenovirus infection investigations in poultry mostly by demonstration of the inclusion bodies within the nucleus of the liver cells as well as particles of virus or antigen identification via microscopy or immunofluorescence test, respectively (Benko *et al*., 2000). More recently, Polymerase Chain Reaction (PCR) has often been used to make a diagnosis of the entire avian adenoviruses groups (Pantin-Jackwood *et al*., 2008). PCR is the preferential direct identification method of FAdVs, whereas serological techniques are of negligible diagnosis significance due to the extensive occurrence of antibodies to the viruses in most birds (Benko *et al*., 2000).

The virus isolation of aviadenovirus employing primary cell cultures of chicken origin supplemented by pathogenicity characterization is of diagnostic significance because of pathogenicity variance within serotypes (Breslin *et al*., 2000). Chicken embryo liver cells diagnostically are preferred because their sensitivity is better than other detection cells (Pantin-Jackwood *et al*., 2008). Then, other tests to characterise them into types are used (Benko *et al*., 2000).

2.8.7 PREVENTION AND CONTROL

Adenovirus infection can be prevented through appropriate disinfection of the barn and equipment, tight biosecurity measures, and good ventilation. Main crucial approach to infection prevention is the maintenance of strict hygiene practices or biosecurity. To avoid vertical transmission, eggs from primary breeding flocks whose progeny have consistently been affected by inclusion body hepatitis (IBH) should not be used for hatching (Pantin-Jackwood *et al*., 2008). However, in countries with high infection pressure (e.g., Australia,

India, Pakistan and Mexico), vaccination has been utilised to manage the disease. Protection is serotype specific (Roy *et al*., 1999).

2.9 IDENTIFICATION CONSIDERATIONS FOR ENTERIC VIRAL PATHOGENS OF POULTRY

Identification and classification of enteric viral pathogens of poultry has conventionally involved electron microscopy, virus isolation as well as immunofluorescence assays (Reynolds and Schultz-Cherry, 2008). Information on enteric viruses are of considerable increase in most recent years with numerous reasons contributing towards such development such as increased awareness that microbes, aside bacteria are a vital element of the aetiology of enteric diseases which have stimulated investigations for further contagious causes amid particular attention given viruses (Saif, 2008). Also, more accessibility to detection equipment was a further motivation; especially through examination of samples by electron microscopy or virus isolation on cell cultures (Decaesstecker *et al*., 1988).

Virus particles are too small to be observed directly by light microscopy because their size is below resolution limit (about 0.3 μm, i.e., 300 nanometres) of the light microscope, although poxviruses, which are within this limit appear to be observable by means of some stain procedures or contrast optics (Murphy *et al*., 2005). However, they can be seen readily in the electron microscope. Many infectious agents can be visualized directly by electron microscopy following clarification and high speed centrifugation of suspect tissue samples. The development of airfuge ultracentrifugation of samples directly onto transmission electron microscopy (EM) grids has improved antigen detection.

Commonly two processes of electron microscopy are of relevance in identification of viruses. Negative staining techniques using phosphotungstic acid and uranyl acetate are used to increase contrast, but in some cases viruses or bacteria may lose identifying projections and may be too widely spaced on the grids or among debris to make positive identification difficult. Negative staining is a rapid technique used to identify viruses or bacteria from an aqueous suspension. Virus or bacteria are absorbed from an aqueous suspension onto specially carbon coated EM grids. The EM grids are stained with aqueous solutions of heavy metals salts which add contrast to any virus or bacteria on the EM grid. Secondly, thin sectioning technique incorporating viruses not yet identified propagated on cultures of cell or fixed sections processed out of infected tissues. However, the principal

diagnostic constraints to the utilization of EM are the issue of sensitivity being comparatively low and non affordability.

Virus identification and propagation are usually carried out employing cell cultures. Utilization of specific pathogen free material is essential for the successful propagation of cells and subsequent use for virus isolation (Schat and Purchase, 1998). The ability to sustain extensive varieties of viral pathogens by primary cell propagation system has enhanced its acceptance as paramount method (Adu, 2011) .Virus isolation is very important and basic for any study on the nature, morphology and properties of a virus. It forms the basis of identification and diagnosis of viral infection and is considered as a classic scheme for their identification, classification and diagnosis (Adu, 2011).

On the other hand, some of the enteric viruses linked to runting-stunting syndrome in poultry especially; chicken parvovirus, astrovirus, as well as avian nephritis virus grow poorly in cell culture, making virus isolation difficult. Hence, it is intricate studying these viruses using usual methods (Tarasiuk *et al*., 2012). Similarly, some viruses may lose identifying projections and may be too widely spaced on the grids or among debris to make positive identification difficult by negative stain electron microscopy (Matsui and Greenberg, 2001). Conversely, molecular procedures are now regularly utilised in recognition of avian viral pathogens. There are many advantages to polymerase chain reaction (PCR) utilization designed for enteric viral pathogen recognition above conventional techniques, including better responsiveness, several viruses' recognition in single sample, virus cultivation not required, the capacity for rapid analysis of huge sample quantities, as well as cheap test cost.

Molecular identification of avian pathogens relies on the recognition of deoxyribonucleic or ribonucleic acids typical of such microbe. Also, analysis of that nucleic acid assessment is utilised in characterization of particular causative microbe. Molecular analytical experiments are on the basis of molecular procedures that may include the PCR and nucleic-acid sequencing. Comparably, the application of real-time PCR has further enhanced enteric viral infections analysis by improved test sensitivity, procedure-time reduction and virus quantification (Smyth *et al*., 2010; Dai *et al*., 2010). Diagnostic tests utilise PCRs for nucleic acid amplification of disease-causing agents that

are present in small numbers, thereby increasing the sensitivity of the test. Then, the amplified DNA can be further analyzed with DNA sequencing.

2.10 SURVEILLANCE AND DISEASE MANAGEMENT

The growing trade in animal and animal products is epitomized by intertwined production, and circulation coordination of food. These developments appear leading to heightened consciousness of possibilities of infection occurrence as well as growing anticipation of veterinary interventions in the promotion of animal welfare and health, food wholesomeness and zoonotic disease control (Rist *et al*., 2014). The entire tasks entail the involvement of ethics pertaining to disease surveillance trend. As a result of these prospects, the application of the principles of disease surveillance, prevention and control is required to meet these trends.

Control and prevention of diseases particularly viral is usually hinged on different approaches which are individually chosen based on virus properties, stability and pattern(s) of spread and disease consequences (Ercsey-Ravasz *et al*., 2012). If accessible, a main important preventive gauge is the widespread vaccination utilization, intended to improve individual or herd immunity (Jones *et al.,* 2008). Also important to control feed and water contaminations are sanitation procedures practiced on farms. In addition, detection and quarantine programs are used in eradicating numerous diseases of domestic animals especially viral. Actualization of plans for disease management is significantly reliant on precise aptitude as regards occurrence as well as spread of diseases.

2.10.1 DISEASE SURVEILLANCE

Regular disease monitoring makes available essential details with methodical assortment, collation as well as study of information on disease occurrence. This facilitates the detection of trends in the circulation of diseases (Jones *et al.,* 2008). Disease monitoring usually encompass techniques that involve population or laboratory observations as well as exposure of disease occurrence by reporting (Bean *et al*., 2013). Additionally, a sense of heightened awareness and dissemination of information is essential to surveillance. In order to present valuable records, information concerning

diseases that are reportable and non reportable are usually pulled together by several countries and these records make available prevention approaches particularly via calculations in relation to expenditure profit equilibrium as well as vaccination efficiency manifestations (Jones *et al.,* 2008). Successive course of eradication is possibly considered and put into operation for a scheduled period via utilization of information based on disease peculiarity, validity of analytical assessment as well as ease of vaccination (Bean *et al*., 2013).

2.10.2 CONTROL OF DISEASE BY MEANS OF SANITATION

Increased accumulation of faeces, body fluffs and other biological products as a result of intensive rearing management of livestock could lead to their contamination with pathogens thereby presenting an avenue for infection of naïve animals (Noah, 2008). In circumventing such infections, rearing schemes whereby livestock housing is cleared and sanitized after a batch of livestock is disposed and before another rearing is undertaken is encouraged. Sanitary procedures mainly appear efficient in management of infection spread through ingestion of contaminated feed and water than air borne infectivity (Chorba, 2001).

The sanitary process of hygienic maintenance of housing as well as tools is of great significance in disease prevention of domestic animals. Nonetheless, diverse viral pathogens differ significantly with respect to susceptibility to sanitizing agents (Taylor *et al.*, 2001). Majority of contemporary sanitizing agents have inactivating effect on nearly every viral pathogen rapidly. Importantly, achievement of sanitary efficiency may be affected considering that viruses deeply entrapped within mucus deposits or faecal materials are unaffected easily (Quinn, 1991).

2.10.3 USE OF QUARANTINE AS A TOOL FOR CONTROL OF DISEASE

Suitable standardized veterinary control systems are significant in monitoring of livestock infections that may occur as a result of transboundary livestock movements and quarantine continues to be the basis of several disease management schemes of livestock. Typically, transborder livestock importation prerequisite is a quarantine interlude which

may be accompanied with analysis of precise causative agents towards aiding the management of particular cause of disease (Fauci and Morens, 2012). Stringent policies geared towards shielding animal production in the midst of escalating relevance of this industry have been implemented through absolute animal importation restriction by regulatory bodies in various countries where improved all-embracing analytic course of actions deliberately targeted at the identification of infection status of animals are implemented (Bean *et al*., 2013).

In addition it is acknowledged nearly every nation state share territorial margins with neighbour nations which may not easily allow movement regulation of humans as well as animals (Jones *et al.,* 2008). Quarantine implementation appears complicated especially in nations with extended territory margins. In overcoming this complexity, information concerning status of animal disease within different nations is interchanged via notifications regulated by the World Animal Health Organization. While an internationally acceptable standard in support of such notification continue to be a dilemma, this structure typically present the prospect of improved awareness as well as vaccination delivery within shared state territory margins (Quinn, 1991). Nonetheless, economic, political as well as social challenges majorly forestall improved quarantine relative to technical know-how (Quinn, 1991).

2.10.4 DISEASE CONTROL THROUGH VACCINATION

Vaccination, which is commonly the main efficient control method aimed at principally reducing the susceptibility of exposed animals to certain viral microbes thereby making such pathogens incapable of partaking in the spread as well as their maintenance in susceptible populace (Fauci and Morens, 2012). Thus, vaccination is different from other means of viral infection control which are centered towards lessening the probability of disease occurrence. The emergence of novel recombinant DNA intended for various uses in vaccination has unlocked a novel field in which concomitant course of disease elimination and vaccination possibly could be carried out (Noah, 2008).

2.10.5 EFFECTS OF ANIMAL REARING METHOD ON CONTROL OF DISEASES

In recent times, methods of livestock rearing scheme have been improved upon with reflective consequences on management of diseases. Conventionally extensive rearing characterised by widespread long-distance ruminant grazing and free-range of poultry are a common practice in virtually all resource-poor as well as a few developed nations (Maclachlan and Dubovi, 2011). Chickens as well as various other livestock majorly had been intensively and semi-intensively reared in practically all nations from time in memorial. Additionally, current animal-welfare interest within intensive components particularly in industrialized countries appears to encourage invigoration of erstwhile traditional rearing schemes while extensive animal production systems such as free-range rearing of poultry and ruminants is very popular in urban and peri-urban areas of developing nations (Noah, 2008).Transmissible microbial diseases frequently play hindering roles in the improvement and viability of rearing intensively. Important characteristics such as confinement as well as inefficient care of a range of livestock obtained from different sources within an intensive rearing scheme (Saif, 2013).

Although an element of existing apprehension over welfare within intensive livestock production structure is disease, not a bit of these crucial distinctiveness associated to these rearing schemes may change due to this important factor for the reason that the financial viability is more connected to the effectiveness of labour as well as feeding than disease. Nonetheless, in attendance are immense advantages of rising production output and fewer overheads when fatalities due to diseases are curtailed. A principal limitation appears to be management and its resolution is necessitated by means of current epidemiologic routines inclusion in livestock personnel instructions (Chorba, 2001). Conventional extensive rearing systems persist in the majority of resource-poor countries for every type of livestock; particularly sub Saharan African countries where primitive rearing systems are common even in the light of considerable increased livestock products demands due to a persistently growing population.

2.11 ECONOMIC IMPORTANCE OF RUNTING-STUNTING SYNDROME IN POULTRY IN NIGERIA

The overall impact of runting-stunting syndrome and associated enteric viruses on poultry health and economics appears not fully appreciated (Long *et al*., 2017). In Nigeria, 22.9% of the gross domestic product is contributed by the agriculture sector (FAOSTAT, 2014) out of which poultry production appreciably add to this number. Nevertheless, veterinary and economic impacts of RSS on the Nigerian poultry industry have attracted inadequate interest probably due to the priority attention given to infections that may include infectious bursal, avian influenza and Newcastle diseases (Oluwayelu *et al*., 2014; Aiki-Raji *et al*., 2015). Additionally, the cost implication of incorrect diagnosis and medication against these viruses may contribute to increased cost of poultry production in the country.

2.12 RUNTING-STUNTING SYNDROME IN POULTRY IN NIGERIA

While there have been reports of enteric viral pathogens linked with RSS in poultry in various countries globally (Koo *et al.,* 2013; Devaney *et al.,* 2016; Kang *et al.,* 2018), there is paucity of information on the occurrence, virus isolation and classification of enteric viral pathogens connected to RSS in Nigerian poultry population. Ighodalo and Akpavie (2008) examined ninety broiler chicks from two farms and reported RSS in the chicks based on histopathological and microbiological examinations. The study revealed the detection of *Streptococcus faecalis, Salmonella gallinarum*, *Klebsiella pneumonia* and *Escherichia coli* in guts of the tested birds and reported that the virus assessment of obtained tissue samples from the chicks was negative. They considered poor growth performance of the birds was as a result of lesion observed in them. A serological survey for CAstV antibodies in twenty five 8-20-week-old indigenous chickens in Ibadan, Oyo State (Oluwayelu and Todd, 2012) using immunofluorescent assay showed low prevalence for this virus. It was concluded in the study that CAstV infections may be a factor responsible for the poor growth performance usually observed in the local chickens. In addition, Oluwayelu *et al*. (2012) using reverse transcriptase- polymerase chain reactions, tested for ANV and CAstV in thirteen pools of samples of intestines and kidneys from apparently healthy indigenous chicken from market and backyard flocks. The study

detected ANV and CAstV RNA in 100% and 84.6% respectively, in gut contents. ANV was found in 3 pools of kidney samples while none was positive for CAstV. This study further showed that poor productivity or performance of Nigerian indigenous chickens may be related to astrovirus infections.

The roles of these viral enteric microbes in RSS-affected commercial chickens and turkeys are poorly implicit in the country. Moreover, these viral enteric microbes in combination with unidentified factors or other microbes could cause RSS. In addition, the early inception of signs may suggest that the agent(s) causing RSS are overpoweringly immunosuppressive (Smyth, 2017) and provide an environment conducive for other pathogens to thrive. Considering the prospect of multiple aetiologies of RSS, developing prophylaxis and control strategies are hindered. The surveillance for enteric viruses associated with RSS in chickens and turkeys in selected states in south west Nigeria may be important in the identification of the aetiological agent(s) and improve the understanding of the pathogenesis of RSS in poultry in the country.

CHAPTER THREE

MATERIALS AND METHODS

3.0 ETHICAL APPROVAL

Ethical approval for this research was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee with reference number (UI-ACUREC/18/0116) (Appendix 1)

3.1 SAMPLE DESCRIPTIONS

3.1.1 Sample location

The study was carried out in Ibadan and Ogbomosho, Ikirun and Abeokuta in Oyo, Osun and Ogun states, respectively, situated in south west Nigeria (Figure 3.1). The South west area is pivotal in commercial poultry activities in Nigeria (Aiki-Raji *et al*., 2015). In addition, the region is the hub of the Nigerian poultry industry serving as crucial points for poultry distribution and trade to and from various parts of the country.

3.1.2 Sources of collected samples

Blood and intestinal contents were obtained from commercial broiler chickens and turkeys with signs of runting-stunting syndrome. The inclusion criteria for sampling include birds with ruffled feathers, splayed legs, wet and poor feather development, poor growth, weakness and culls. A total of 261 birds were sampled consisting of 164 broiler chickens and 97 turkeys in Ogun, Osun and Oyo States (Table 3.1). These birds were rejects from six commercial poultry hatcheries and/or culled from different flocks in ten poultry farms in the selected states (Table 3.2).

Figure 3.1: Map of Nigeria showing collection sites (Source of the map: User:Gar3th [CC BY 3.0 (https://creativecommons.org/licenses/by/3.0)], from Wikimedia Commons (https://commons.wikimedia.org/wiki/Atlas_of_Nigeria#/media/File:Nigeria_states.png)

Table 3.1: Collection sites of blood samples from broiler chickens and turkeys

Location	Broilers	Turkeys
Oyo		
Ibadan	33	63
Ogbomosho	44	0
Ogun		
Abeokuta	67	0
Osun		
Ikirun	20	14
Total	164	97

Table 3.2: Distribution of samples based on collection source

	Broilers	Turkeys	Total
Hatchery	104	51	155
Farm	60	46	106
Total	164	97	261

The 164 broiler chickens consisted of six 14-week old and 158 day-old birds while all the turkeys were day-old poults. The day-old broiler chickens and turkeys were all runts and the older chickens were undersized (Figure 3.2).

3.1.3 Design and period of study

This study was carried out using purposive sampling method to focus on commercial broiler chickens and turkeys with runting-stunting syndrome in Oyo, Ogun and Osun states, south west Nigeria. Total sampling of birds with signs of runting-stunting syndrome was carried out. Sample collection was conducted within a six months period between November 2017 and April 2018.

3.1.4 Sample collection, storage and transportation

3.1.4.1 Blood samples

About 1.5 milliliters of blood was aseptically obtained by venopuncture from every commercial broiler chicken and turkey with signs of runting- stunting syndrome (using sterile 2 mL syringe fitted with 21G x 1½" needle) through the jugular vein. The sample was spun at 3,000 rpm for about five minutes. The separated sera were then decanted into sterile well-labelled Eppendorf tubes. Heat inactivation of sera was carried out at 56 °C for 30 minutes. The sera were subsequently kept at - 20 °C prior to transportation by courier, over ice, to the avian virology laboratory of the Agri-Food and Biosciences Institute (AFBI), Belfast, United Kingdom where they were kept at -80 °C pending examination. A total of 261 serum samples comprising 164 from broilers (158 day-old and 6 fourteen weeks old) and 97 day-old turkeys were obtained from poultry hatcheries and /or farms situated in Oyo (Ogbomosho and Ibadan), Ogun (Abeokuta) and Osun (Ikirun) states, south west Nigeria.

Figure 3.2a: Runted day old broiler chick with signs of paralysis and ruffled feather

Figure 3.2b: Runted turkey poults with poor feather development, and paralysis

Figure 3.2c: Stunted 14-week old broilers

3.1.4.2 Intestinal contents

The sampled birds were immediately euthanized through cervical disarticulation before samples were obtained from them. Intestinal contents were collected from each commercial broiler chicken and turkey with signs of runting- stunting syndrome. A total of 261 intestinal contents comprising 164 from broilers (158 day-old and six fourteen weeks old) and 97 day-old turkeys were obtained from poultry hatcheries and /or farms situated in Oyo (Ogbomosho and Ibadan), (Abeokuta) Ogun and (Ikirun) Osun States, south west Nigeria. With the aid of forceps, feathers were scrapped away from the chest and abdomen area of the bird to expose the sternum. The bird was then opened up with a pair of scissors and the intestine with intestinal contents removed. Each sample was collected into well-labelled sterile sample bottle and stored at -70 °C prior to shipment by courier, over ice, to the avian virology laboratory of the Agri-Food and Biosciences Institute, United Kingdom and stored at -80 °C until analyzed.

SECTION ONE

3.2 SERODETECTION OF ASTROVIRUSES IN COMMERCIAL BROILER CHICKENS AND TURKEY POULTS WITH RUNTING-STUNTING SYNDROME

3.2.1 INDIRECT IMMUNOFLUORESCENCE ASSAY

3.2.1.1 Cleaning and sterilization of multispot slides

A 5-10% 7X phosphate free detergent (Thermo Fisher Scientific) was made in hot water. Multispot slides were inserted in a metal slide basket and the basket immersed in the detergent solution. The slides were then rinsed in two changes of cold distilled water and allowed to dry. Each individual multispot slide was held up to the light and examined for dirt or smudges. If necessary, the slide was polished with tissue paper. Then, the slides were assembled in groups of 30 and wrapped in aluminium foil and subsequently incubated for 6 hours at 160 °C for sterilization, then cooled and kept wrapped in foil until required. To use, multispot slide (with number side up) was placed with the aid of sterile forceps on sterile plastic Petri dish in a Micro flow cabinet.

3.2.1.2 Preparation of Phosphate buffered saline, pH 7.2

Distilled water (1 L) was poured into a sterile container and positioned on magnetic agitator set to around 200 rpm. Ten tablets of phosphate buffered saline were gradually added to container whilst agitating and stirred until dissolved entirely. The pH was adjusted accordingly. The contents of the container were then dispensed into 100 mL graduated bottles. The bottles were capped and labelled with date and contents. The sealed bottles were sterilized by autoclaving for 20 minutes at 121 °C and then stored at room temperature.

3.2.1.3 Tissue culture preparations of antigen infected cells on multispot slides

Cultures of chicken hepatocellular carcinoma stimulated by LMH cells (Kawaguchi *et al*., 1987) separately infected using three different antigens; avian nephritis virus serotype1, chicken astroviruses, CAstV 612 and CAstV 11672 were used.

Forty mL of cells were dispensed into each Petri dish containing three multispot slides. To monitor antigen/cell growth, a slide was fixed by removing it from the Petri dish in a Micro flow cabinet Class II using sterile forceps. The slide was then dipped into a Petri dish containing phosphate buffered saline (PBS) followed by two more dishes containing acetone. Finally, the multispot slide was placed in a Petri dish containing acetone for 10 minutes, lifted out of the acetone and left to dry completely on filter paper. The spread of positive cells was monitored by immunofluorescent assay (IFA) using a primary serum specific to each of the three antigens. Once the test slide showed a satisfactory positive quantity in cells, remainder of such used multispot slides was harvested. The antigen-infected multispot slides were then stored at -20 °C in a plastic box containing some silica gel and the box sealed with insulating tape.

3.2.1.4 Testing of serum by indirect immunofluorescence assay

The indirect immunofluorescence assay (IIF) was carried out as earlier depicted (Todd *et al*., 2009) with collected sera using antigen-infected multispot slides. The three specific antigens used in this study were avian nephritis virus serotype 1, as well as strains 612 and 11672 of chicken astrovirus shown to have genetic and antigenic distinction. The required number of fixed, antigen-infected multispot slides were removed from the refrigerator and left at room temperature for one minute to allow moisture on the slides dry up. Using a PAP pen, each well of the multispot slides were carefully waxed round and placed on staining rack in humidified chamber (plate 3.1).

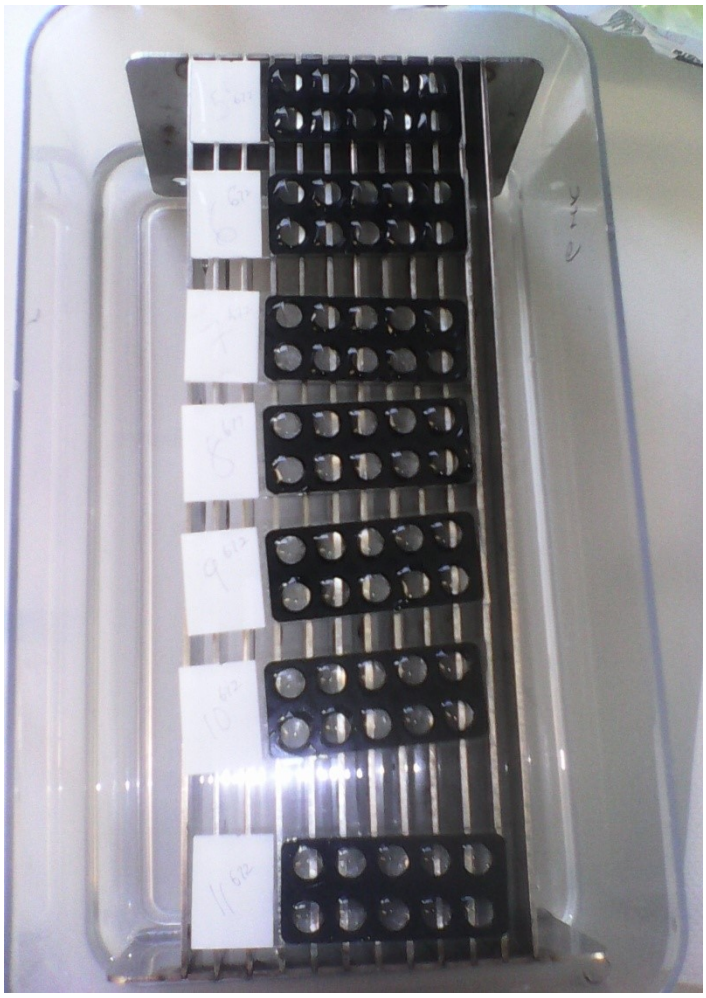


Plate 3.1: Multispot slides with test samples placed on staining rack in humidified chamber

Ten micro litres of serum was added to ninety micro litres PBS in Eppendorf tubes to make a1:10 dilution of each test serum. 30 µl of each diluted serum was applied in duplicate to the appropriate multispot well. Positive and negative controls were also added. For IIF test for ANV, ANV-1 positive serum was used as positive control. While for IIF test for CAstV, positive serum for CAstV 612 or CAstV 11672 was used. Negative control was PBS. The chamber was covered and incubated for 1 hour in a five percent carbon dioxide incubator set for 37 °C. This was followed by removal of chamber from incubator and checked that none of the test wells had merged, after which the multispot slides on the staining rack got rinsed thrice using PBS, and excess PBS from multispot drained and the slide returned to rack in humidified chamber.

The secondary antibody-conjugated, fluorescein isothiocyanate (FITC), rabbit anti-chicken immunoglobulin G (Sigma, USA) diluted in PBS in ratio of 1:80 was used. The bound antibody on each well of the multispot was then reacted with 30 µl of diluted FITC-conjugated secondary antibody for 37 °C at an hour. Afterward, the chamber was brought out of the incubator and the multispot slides on the staining rack were rinsed with PBS three times. The excess PBS was drained using filter paper.

For mounting of the multispot slides, the required number of rectangular cover slips was placed on paper towel. A line of Citifluor mount ant medium (Citifluor Ltd, UK) was applied down the middle of the cover slip. The multispot were removed from the rack and the cell side of the slide placed onto the cover slip with the mount ant medium. The weight of the multispot was allowed to spread the Citifluor evenly over the cells and any excess was absorbed by tissue paper. The multispot slide was lifted and the back dried with fresh tissue paper before being placed in a cardboard slide holder. To view under the fluorescent microscope (Leitz Germany), immersion lubricant in right quantity was placed on each well of the multispot and cells examined at x40 magnification. The aim is to identify wells that give bright, specific fluorescent staining in infected cells. Samples considered positive showed intracytoplasmic immunofluorescent staining.

3.2.2 Statistical Analysis

The analyses of obtained data were carried out using the GraphPad Prism (version 7.0) statistical software. Differences in detected astrovirus antibodies between broiler

chickens and turkey poults, and day old and adult chickens were evaluated using chi-square (X^2) test. The level of significant association was determined at a value of $\alpha_{0.05}$.

SECTION TWO

3.3 ISOLATION OF ENTERIC VIRUSES ASSOCIATED WITH RUNTING-STUNTING SYNDROME IN COMMERCIAL BROILERS AND TURKEYS

3.3.1 PREPARATION OF REAGENTS

(A) Phosphate buffered saline, pH 7.2

Distilled water (1 L) was added to a sterile container and positioned on magnetic agitator set to around 200 rpm. Ten tablets of phosphate buffered saline were gradually added to the container whilst agitating and stirred until dissolved entirely. The pH was adjusted accordingly. The contents of the container were then dispensed into 100 mL graduated bottles. The bottles were capped and labelled with date and contents. The sealed bottles were sterilized by autoclaving for 20 minutes at 121 °C and then stored at -20 °C.

(B) Preparation of transport media

Solution of chilled phosphate buffered saline was enhanced by addition of 100,000 µg/mL of streptomycin, 100,000 unit/mL penicillin as well as 100, 000 µg/mL amphotericin B.

3.3.2 PREPARATION OF 10% (W/V) INTESTINAL CONTENT SUSPENSION USING THE TISSUELYSER II APPARATUS

The intestine and intestinal contents were defrosted at room temperature. In a safety cabinet, tissue was placed onto a tissue culture dish. Fat and connective tissue were trimmed off using forceps and scalpel.

Two sterile metal beads were placed into a 2 mL micro centrifuge tube and 1 mL of transport media also added. Approximately 0.2g of intestine and intestinal contents was weighed and transferred into the tube. A lid was secured on the tube and positioned for 5 minutes on ice. The tubes were then placed in TissueLyser II (Qiagen, Germany) adapter set (2 X 24) ensuring that the tubes were balanced and operated for 45 seconds at 30Hz. The adapter set was uncoupled, alternating tube racks so that the racks next to the TissueLyser II were now outer most, and then coupled back. It was then passed through

another run of 45 seconds at 30 Hz. The tubes were then placed on ice until the froth settled.

With a sterile Pasteur pipette, the homogenate was carefully transferred into a labelled microcentrifuge tube and the micro centrifuge tube rinsed by means of a milliliter of transport media and then poured into the microcentrifuge tube. This gave a final 10% (w/v) gut content suspension. The gut content suspension was then spun on 3,000 rpm for 30 minutes at 4 °C to remove cell fragments and bacteria. The resulting supernatant was removed with a sterilized Pasteur pipette; ensuring that no debris was picked, aliquots were made into pre-labelled vials and stored at -80 °C until tested.

3.3.3 CELL CULTURE PREPARATION

3.3.3.1 Preparation of cell culture reagents

(A) wash solution

Dulbecco's PBS (D-PBS) is prepared devoid of magnesium as well as calcium (D-PBSA). This is usually employed as a trypsin solvent as well as rinse solution.

Preparation and sterilization of wash solution

Ultrapure water (1 L) was poured in a sterile container and positioned on a magnetic agitator set to around 200 rpm. Ten tablets of D-PBS A were slowly added to container while mixing and stirred until completely dissolved and instantly subjected to filtration to prevent precipitation or pollution by constituent or microbes, respectively. The pH was accordingly adjusted. Contents of the container were then dispensed into 100 mL graduated bottles. 225 µl of Gentamycin was added to every 100 mL of the D-PBSA solution. The bottles were capped and labelled with date and contents. The sealed bottles were sterilized by autoclaving for 20 minutes at 121 ° and then stored at room temperature.

(B) 0.05% trypsin solution

A preparation of 0.25% trypsin solution was made by adding 2.5 g of trypsin to one litre of sodium chloride solution. The trypsin was stirred for an hour at room temperature and afterward sterilized by filtration. This was then dispensed into 10 to 20mL aliquots

and stored at -20°C. Subsequently, the trypsin (025%) was thawed and diluted 1:5 in D-PBSA to obtain 0.05% trypsin solution.

(C) Growth medium

Solution of Medium 199 was enhanced by addition of foetal calf serum (10%) as well as Gentamycin (100µg/mL).

(D) Maintenance medium

Solution of Medium 199 was enhanced with 2% foetal calf serum and (100 µg/mL) Gentamycin

3.3.4 PREPARATION OF PRIMARY CHICK EMBRYO LIVER CELL CULTURES

Specific pathogen free chicken embryos (14-16 day old) were chilled at -80 °C for 10-15 minutes to kill embryos and prevent bleeding. The eggs were spaced out on the egg tray before being placed into the freezer to allow the cold air to circulate around the eggs. After chilling, the egg surfaces were sterilized by spraying 1% Virkon solution to reduce contamination. To maintain sterility, all further work was carried out in a Laminar flow work station. Using curved scissors, shells around the air space of the eggs were removed. The embryos were taken out from the eggs using sterile surgical hook and placed on sterile Petri dish. To ensure the embryo is dead before the body was dissected, the head was cut off. With fine forceps, the feathers from the chest and abdomen area were scrapped away to expose the V shape of the sternum. The embryo was then cut at the bottom of the rib cage and the liver removed. The gall bladder was separated from the removed liver because bile is toxic to tissue culture. Blood and feathers were washed off the collected livers by dipping into two universal bottles containing wash solution (PBS 'A' and 2% Gentamycin); this was done by allowing the livers sit in the wash solution for few minutes to allow the residual blood leach out.

The washed liver samples were then chopped finely with a scalpel; ensuring not to use a cutting motion as this will tear the tissue and encourage the unwanted growth of fibroblast cells. The liver homogenate was transferred to the trypsinization flask and 100 mL of wash solution added after which it was positioned on a stirring base within an incubator at 37 °C and whisked at a medium speed for 2-3 minutes to wash and remove

any blood or fibrous material. About 100 mL trypsin (0.05%) was put in the liver homogenate and stirred at 37 °C at a medium speed at 2-3 minutes interval. The supernatant was collected at each interval and poured into sample bottle containing 10 mL of foetal bovine serum placed in an ice bath (the serum and ice will stop the enzyme reaction from further digesting the cells). The addition of trypsin and supernatant collection was repeated until all the tissue is digested. The supernatant was subsequently spun (4 °C) at 112 x g for 15 minutes obtain cell pellets.

The pellets were re-suspended in 20 mL of M199 growth media and centrifuged at 112 x g for 5 minutes. The pellets at this point were the packed cell volume (PCV). The volume of the PCV was read and diluted in growth media accordingly. The diluted cell suspension was then gassed with carbon dioxide until saturated and the media changed from red colour to yellow or orange. This was then made into aliquots in roller tubes and kept warm for up till 3 days at 37 °C to permit development of a monolayer after which the growth media was changed to maintenance media to maintain the culture for approximately 5-7 days.

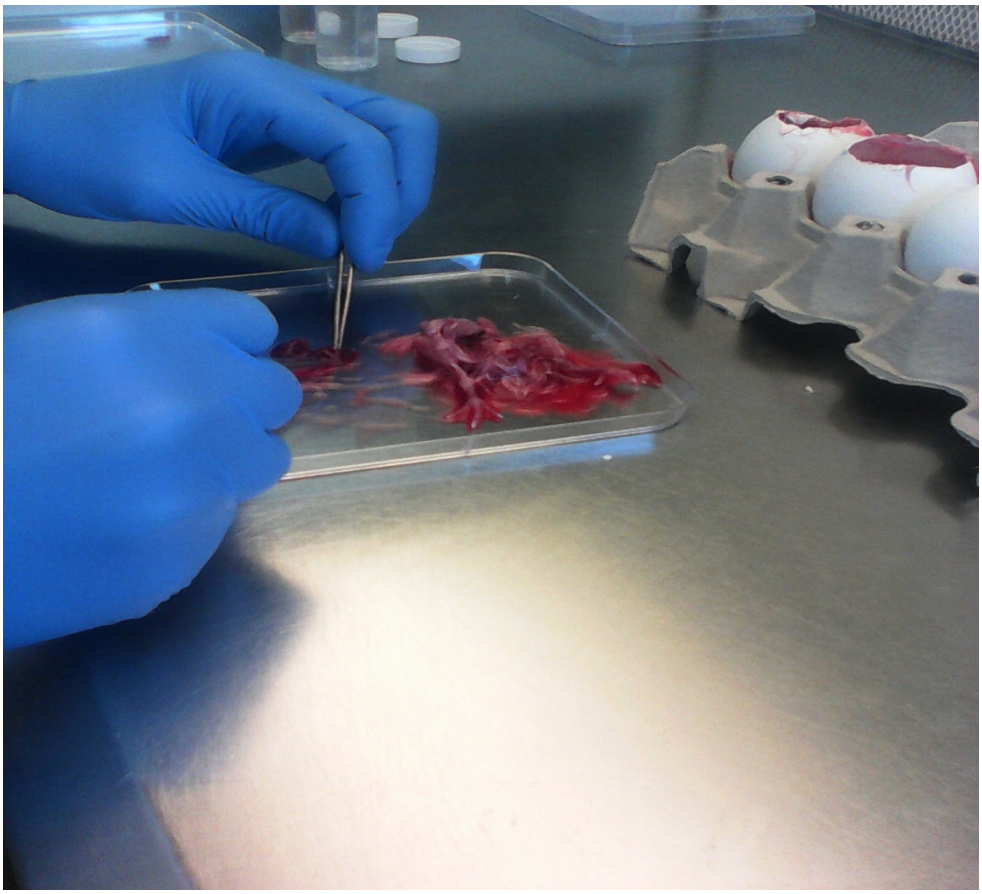


Plate 3.2: Slicing of liver samples from specific pathogen free (SPF) chicken embryos

3.3.5 VIRUS ISOLATION

One hundred mL of the prepared 10% (w/v) gut content suspension was injected in the primary chicken embryo liver cell culture in duplicate, and subjected to 72 hours incubation at 37 °C. The infected monolayer as well as controls was inspected after 72 hours. Where cell degeneration was not seen or is unclear, the cells and fluids from the containers were collected and a blind passage made onto a fresh monolayer. Observation of cytopathic effects following 1 to 3 sub cultures indicates possible detection of virus. Cell cultures were frozen and thawed three times and stored at -80 °C.

Plate 3.3: Inoculation of 10% intestinal content homogenates onto cell culture

3.3.6 IDENTIFICATION BY NEGATIVE CONTRAST ELECTRON MICROSCOPY

3.3.6.1 Preparation of sample suspension

The sample was spun in a micro centrifuge at 13,000 rpm for 30 minutes and the supernatant discarded. Resulting pellet was re- dissolved in 50 µl filtered water and mixed by using a vortex mixer for 30 seconds.

3.3.6.2 Negative staining of test samples and identification

A drop (50 µl) of each test sample to be stained was placed on wells of multispot slide placed in a staining dish. A carbon coated grid, with the carbon coated surface facing downwards was placed on the surface of the drop of test sample. This was kept for 10 minutes incubation on the bench. The grid was lifted with forceps and excess material drained with filter paper. 50 µl of 4% ammonium molybdate was placed on another well of the multispot slide and the carbon grid placed, with the carbon coated surface on the surface of the drop of 4% ammonium molybdate. This was stained for 60 seconds and the grid lifted with forceps and excess material drained with filter paper and further dried out for 10 minutes. Dried grid was afterward examined in the electron microscope.

3.3.7 Statistical Analysis

The analyses of derived data were carried out by means of the GraphPad Prism (version 7.0) statistical software. Variations in isolated and identified enteric viruses between broiler chickens and turkey poults, and day old chicks and adult broilers were assesse d using chi-square (X^2) test. The level of significant association was determined at a value of $\alpha_{0.05}$.

SECTION THREE

3.4 MOLECULAR DETECTION OF ENTERIC VIRUSES ASSOCIATED WITH RUNTING-STUNTING SYNDROME IN COMMERCIAL BROILERS AND TURKEYS

3.4.1 Nucleic acid extraction

The enhanced high-throughput workstation, MagNA Pure 96 Systems® automated for purification of nucleic acids allows for considerably improved efficiency and output (Figure 3.3).

Automated extraction of RNA and DNA viral nucleic acids was carried out by the MagNA Pure 96 robotic workstation directly from the 10% intestinal content suspensions using the Viral Nucleic Acid LV Kit (Roche, Britain) following information provided in the manufacturer's manual. A negative extraction control, phosphate buffered saline (pH 7.2); instead of test sample supernatant as well as a positive control from identified virus stock, was incorporated in every extraction set of samples. Obtained nucleic acids were kept pending usage at -80 °C.

Figure 3.3: The MagNA Pure 96 (Roche, UK) robotic workstation

3.4.2 CHICKEN ASTROVIRUS AND AVIAN NEPHRITIS VIRUS CAPSID PROTEIN RT-PCR ASSAYS

Bearing in mind that the main hyper variable area of astrovirus is the capsid protein which determines its antigenicity (Krishna *et al*., 2005; Smyth *et al*., 2012), capsid gene detection RT-PCR was conducted with the RNA extracted from the 10% (w/v) suspensions obtained from chickens and turkeys. Using forward and reverse primers sited within the intergenic area linking ORFs 1b and 2 as well as just at the end of the capsid gene, respectively, a fragment of approximately 2.2 kb capsid gene (ORF2) product from CAstVs was amplified (Smyth *et al*., 2012). The AffinityScript One-Step RT-PCR Kit (Agilent Technologies, USA) was used according to test specifications (Table 3.3) in a 25 µl PCR reaction volume in a Veriti thermo cycler. The reaction was set on track with a cycle of RT lasting 30 minutes at 45 °C, preliminary denaturation running at 94 °C for 2 minutes then, 40 rounds of amplification (94 °C running for 15 seconds, 52 °C running for 30 seconds, 68 °C running for 150 seconds) and a concluding extension running for 7 minutes at 68 °C.

Similarly, for the ANV assay, using primers previously described (Todd *et al*., 2011); a fragment of about 2.2 kb, enclosing ANV capsid genetic material was amplified. The reaction conditions for ANV capsid PCR were similar to that of CAstV except for the annealing temperature (56 °C for 30 seconds). The primer sequence details for both assays are shown in Table 3.4.

Table 3.3: AffinityScript One-Step RT-PCR Kit recommended assay concentration

Reagent	µl/reaction
2x RT-PCR Master Mix	12.5
Primer (forward)	1.0
Primer (reverse)	1.0
Enzyme	0.5
Diethyl pyrocarbonate-treated water	7.5
Total Master Mix	22.5

Table 3.4: Primers and nucleotide sequences applied to the identification of enteric viruses

Enteric viral pathogen	Primers	Nucleotide sequence	Expected band size (bp)
Chicken astrovirus	CAstV pre cap	TAGAGGGATGGACCG AAA TAT AGC AGC	2,200
	CAstV post cap	TGC AGC TGT ACC CTC GAT CCTA	
Avian nephritis virus	ANV cap F	GAC TTC TAC AGA AAA ATC TGG TGA G	2,200
	ANV cap R	TTC CTG TAC CCT CGA TGC TAC TCG	
Avian rotavirus	Rot P1	GGG CGT GCG GAA AGA TGG AGA AC	630
	Rot P2	GGG GTT GGG GTA CCA GGG ATT AA	
Avian reovirus	Reo P1F	AGT ATT TGT GAG TAC GAT TG	1,100
	Reo P4R	GGC GCC ACA CCT TAG GT	
Turkey astrovirus 1	TAstV-1 F	AGCTYATGMGGTTCTTTCTTCTYG	251
	TAstV-1 R	GATGGTGGGTAGCCTATTGTGTTC	
Turkey astrovirus 2	TAstV-2 F	TGGACCGACCCRRTTTTYACCA	911
	TAstV-2 R	GGCCCGACYTCAGGMAGTTGT	
Fowl adenovirus	Adenovirus Hex A	CAA RTT CAG RCA GAC GGT	900
	Adenovirus Hex B	TAG TGA TGM CGS GAC ATC AT	

3.4.3 DETECTION OF NON-STRUCTURAL PROTEIN 4 OF AVIAN ROTAVIRUS

Conventional RT-PCR was conducted for avian rotavirus (AvRV) using primers (Table 3.4) to facilitate amplification of the non-structural protein (NSP) 4 of group A AvRv (Koo *et al*., 2013). The AffinityScript One-Step RT-PCR Kit was used according to test recommended concentration (Table 3.3) to obtain a total volume of 22.5 µl of Master Mix for each reaction. Subsequently, 22.5 µl of the mix was pipette into every PCR tube. Then using a new pipette tip each round, 2.5 µl of test RNA was dispensed into each tube, after which the tubes were firmly covered and briefly vortexed to ensure proper mixing and next, spun down on a bench top mini centrifuge.

The 25 µl volume reaction was carried out in a Veriti PCR thermocycler (Thermo Fisher Scientific) with thermal cycling setting of a cycle of reverse transcription (45 °C lasting 30 minutes), preliminary denaturation (94 °C lasting 2 minutes) followed by 40 rounds of amplification that includes denaturation (94 °C lasting 15 seconds), annealing (58 °C lasting 30 seconds), extension (68 °C lasting 45 seconds) and a concluding extension of 68 °C lasting 7 minutes.

3.4.4 DETECTION OF THE SIGMA C PROTEIN OF AVIAN REOVIRUS

Conventional RT-PCR using primers (Table 3.4) targeting fraction of the ORF of the sigma C protein predetermined by avian reovirus S1 fragment (Kant *et al*., 2013) was conducted by means of AffinityScript One-Step RT-PCR Kit following recommendations to obtain a reaction volume of 22.5 µl Master Mix for every run. Subsequently, 22.5 µl of the mix was pipette apiece in PCR tubes. Then using a new pipette tip each round, 2.5 µl of test RNA was dispensed into each tube, after which the tubes were firmly covered and briefly vortexed to ensure proper mixing and next, spun down on a bench top mini centrifuge. The reaction cycling conditions were similar for AvRv.

3.4.5 DETECTION OF POLYMERASE GENE OF TURKEY ASTROVIRUSES 1 AND 2

Reverse transcription PCRs were performed using primers (Table 3.4) targeting the polymerase gene (ORF 1B) of turkey astrovirus (TAstV) type 1 as well as TAstV type 2 (Day *et al*., 2007) with AffinityScript One-Step RT-PCR Kit according to recommendations to obtain a reaction volume of 22.5 µl Master Mix for every run. Afterward, 22.5 µl of the mix was pipette apiece in PCR tubes. Then using a new pipette tip each round, 2.5 µl of test RNA was dispensed into each tube, after which the tubes were firmly covered and briefly vortexed to ensure proper mixing and next, spun down on a bench top mini centrifuge. The thermal cycling conditions started with a cycle of reverse transcription (45 °C lasting 30 minutes), preliminary denaturation (94 °C lasting 30 minutes) followed by 40 rounds of amplification that includes denaturation (94 °C lasting 15 seconds), annealing (55 °C lasting 30 seconds), extension (68 °C lasting 45 seconds) and a concluding extension of 68 °C lasting 7 minutes.

3.4.6 DETECTION OF NON-STRUCTURAL PROTEIN GENE OF PARVOVIRUSES

Parvoviruses encode a non structural (NS) gene within left half of the coding sequence that predetermines a minute amount of virus gene and proteins responsible for structuring and replication, respectively that occupies the right half (Cotmore and Tattersall, 2006). The genome sequence of the non structural genetic material is shown to be much conserved within these viruses as well as frequently employed in aiming for PCR analysis (Soares *et al*., 1999). Thus, the extracted nucleic acid from the 10% suspensions from commercial broiler and turkey samples were subjected to PCR in line with the method expressed by Zsak *et al*. (2008) to identify parvoviruses within poultry samples.
The highly conserved NS gene amplification was achieved by PCR by means of primers PVF1(TTCTAATAACGATATCACTCAAGTTTC)andPVR1(TTTGCGCTTGCGGTGA AGTCTGGCTCG) for forward and reverse, respectively previously described (Zsak *et al*., 2009), and Hot Start Taq PCR Master mix Kit (Qiagen, Germany) according to assay recommendations (Table 3.5) to obtain a reaction volume of 22.5 µl Master Mix for every run. Subsequently, 22.5 µl of prepared mix was pipette apiece into PCR tubes. Then using a new pipette tip each round, 2.5 µl of test DNA was dispensed into each tube, after which

the tubes were firmly covered and briefly vortexed to ensure proper mixing and next, spun down on a bench top mini centrifuge. The experiments were conducted in Veriti thermo cycler with cycling conditions of a cycle of preliminary denaturation running for 15 minutes at 95 °C, then thirty five rounds comprising denaturation, annealing and extension of 94 °C running for 30 seconds, 55 °C running for a minute as well as 68 °C running for a minute, respectively, as well as a last extension running for 7 minutes at 68 °C.

Table 3.5: Hot Start Taq PCR Kit recommended assay concentration

Reagent	µl/reaction
Taq PCR Master Mix	12.5
Forward primer	2.5
Reverse primer	2.5
Diethyl pyrocarbonate-treated water	5.0
Total Master Mix	22.5

3.4.7 DETECTION OF HEXON GENE OF FOWL ADENOVIRUS

The adenovirus hexon genetic material was amplified through PCR using suitable primer set (Table 3.4) previously described (Meulemans *et al*., 2001) and Hot Start Taq PCR Master mix Kit (Qiagen, Germany) according to assay recommendation (Table 3.5) to obtain a reaction volume of 22.5 µl Master Mix for every run. Subsequently, 22.5 µl of prepared r mix was pipette apiece into PCR tubes. Then using a new pipette tip each round, 2.5 µl of test DNA was dispensed into each tube, after which the tubes were firmly covered and briefly vortexed to ensure proper mixing and next, spun down on a bench top mini centrifuge.

The experiment was conducted by means of Veriti thermo cycler with cycling conditions of a cycle of preliminary denaturation(95 °C; 15 minutes), then 35 rounds comprising three phases: denaturation (94 °C lasting 30 seconds), annealing (62 °C lasting 30 seconds) and extension (72 °C lasting 30 seconds) as well as 7 minutes of concluding extension at 72 °C.

3.4.8 EVALUATION OF CONVENTIONAL RT-PCR AND PCR PRODUCTS

3.4.8.1 Preparation of agarose gel

Preparation of 1.5% agarose gel was carried out by pouring 1.5 grams agarose powder in to 100 milliliters of tris-acetate ethylene diamine tetra acetic acid (TAE) buffering solution in a conical flask. The suspension was gently mixed and heated for about 2-3 minutes in a microwave oven with swirling of the flask at intervals to ensure proper melting of the suspension. The molten agarose was allowed to cool for a short time and five microlitres of ethidium bromide was included and gently mixed. A casting tray was prepared by sealing the ends and an appropriate comb fixed on the tray. The molten agarose was then poured in the gel casting tray with gel comb in place, being careful not to make any bubbles in the gel and allowed to solidify.

3.4.8.2 Gel electrophoresis

Once casted gel solidifies, inserted comb in the tray was carefully detached as well as the sealing at the ends of the casting tray. The casting tray with the gel was positioned

in a tank containing sufficient buffer solution to ensure that the gel was completely submerged and set ready for electrophoresis. The orientation of the gel was such that the ends flanking the negative electrode of the power pack bear the wells. Subsequently, Ready-load DNA (1kb plus) ladder (Invitrogen, USA) was pipette in the first well of gel to serve as molecular marker. Next, DNA loading dye (Gel Pilot, Qiagen) mixed with the PCR products as well as positive and negative controls in ratio 1:5 was loaded in other wells of the gel. The lid was placed on the electrophoresis tank and power system was operated for 1 hour at 100 volts.

After the run, the casting plate bearing the gel was picked out of the tank. The gel was then viewed under ultraviolet trans-illumination with the molecular marker as size estimation guide.

SECTION FOUR

3.5 QUANTIFICATION OF CHICKEN PARVOVIRUS, AVIAN NEPHRITIS VIRUS AND CHICKEN ASTROVIRUS BY REAL-TIME PCR AND RT-PCR

3.5.1 CHICKEN ASTROVIRUS AND AVIAN NEPHRITIS VIRUS

An operational template of the reaction was prepared to show the positions of RNA samples and the controls on PCR plate of 96 wells. Frame Star Fast plate 96-well semi-skirted, low volume PCR plates (4titude, UK) were used. Real-Time Reverse Transcriptase Polymerase Chain Reactions (RT-qPCR) for CAstV as well as ANV were performed as previously described (Smyth *et al*., 2010). Sample testing for RT-qPCR was carried out in triplicate with a total volume of 20 µl for each reaction replicate. The probe with a working final dilution concentration of 120 nM as well as the primers (reverse and forward) to 400 nM concentration were mixed in a ratio of 1:1:1 to give a probe/ primers (PPP) mix. The specifications of primer sequences as well as probes (TaqMan-labeled) bearing non fluorescent fractions (minor groove binder) used in these assays are presented in Table 3.6.The Ambion AgPath-ID real time One-step RT-PCR kit (ThermoFisher Scientific, USA) was used in recommended assay concentrations as shown (Table 3.7).

The plate was placed on a holder and 18 microlitres of Master -Mix was pipette apiece in wells of the microplate. Then two microlitres of test RNA was included in triplicate. Also, positive extraction, positive PCR, negative extraction and negative PCR controls were added in triplicates. The plate was firmly sealed with PCR/real-time PCR compatible MicroAmp optical adhesive film and the sealed plate vortexed briefly and then centrifuged (4 °C) for a minute at 3000 rpm.

The experiment was performed in a Fast plate (7500) Real Time PCR system (ThermoFisher Scientific, USA) with initial RT phase of 10 minutes set for 45°C, followed by a preliminary phase of denaturation for 10 minutes set for 95°C, then, 40 rounds comprising denaturation for 15 seconds at 95°C, and subsequent aligning of primers plus amplification of genetic materials achieved by 60°C temperature maintenance lasting 45 seconds. At amplification phase, fluorescence analyses were

obtained. As the experiments get to the exponential phase preceding that of linear phase, the cycle thresholds (C_T) were positioned. Amplification of a section in the pre capsid area of seventy base pair magnitude was achieved in CAstV test as that of untranslated area (3') of fifty six base pair within the genome of ANV was accomplished by test for ANV. Using logarithmic (log_{10}) scales, virus RNA quantity in every sample tested was calculated for both CAstV and ANV assays using the procedure according to Smyth *et al*. (2010);

Log viral copy number = $(C_{T\ Test} - C_{T\ Yintercept}) / M$

In which, $C_{T\ Test}$ is value of C_T for each sample tested, $C_{T\ Yintercept}$ is the slope cutoff on C_T axis and M is the slope.

The mean logarithm value for each test sample was then determined.

Table 3.6: Primers and nucleotide sequences for CAstV and ANV RT-qPCR assays

Primers	Nucleotide sequence (5' – 3')
CAstV forward	GCYGCTGCTGAAGAWATA CAG
CAstV reverse	CATCCCTCTACCAGATTTTCT GAA A
Probe	6-FAM-CAG AAG TCG GGC CC-MGB
ANV forward	GTA AAC CAC TGG YTG GCT GAC T
ANV reverse	TAC TCG CCG TGG CCT CG
Probe	6-FAM-CAG CAA CTG ACT TTC-MGB

Table 3.7: The RT-qPCR Master Mix for ANV and CAstV assay

Reagent	µl/reaction
2x RT-PCR buffer	10.0
Nuclease-free water	6.2
RT-PCR enzyme mix	0.8
PPP mix	1.0
Total master mix	18.0

3.5.2 CHICKEN PARVOVIRUS

An operational template of the reaction was prepared to show the positions of DNA samples and the controls on PCR plate of 96 wells. Frame Star Fast plate 96-well semi-skirted, low volume PCR plates (4titude, UK) were used. The qPCR for ChPV was executed as described earlier (Zsak *et al*., 2009) that amplify identified strains of chicken and turkey parvovirus. The qPCR experiments were in triplicate for each test sample in a 20 µl volume for each experiment replicate. The probe with a final dilution concentration of 120 nM as well as the primers (reverse and forward) to 400 nM concentration were mixed in a ratio of 1:1:1 to give a probe/ primers (PPP) mix. The Brilliant UltraFast qPCR kit (Agilent Technologies, USA) was used in recommended assay concentrations (Table 3.8).

Table 3.8: Real-time PCR Master Mix for ChPV assay

Reagent	µl/reaction
Master Mix	10.0
Rox dye	0.3
PPP mix	1.5
Nuclease-free water	6.2
Total master mix	18.0

The plate was positioned on a holder and 18microlitres of Master Mix was pipette apiece in wells of the microplate. Then two µl of test DNA was added in triplicate. Also, positive extraction, positive PCR, negative extraction and negative PCR controls were added in triplicates. The plate was firmly sealed with PCR/real-time PCR compatible MicroAmp optical adhesive film and the sealed plate vortexed briefly and subsequently spun (4 °C) at 3000 rpm for one minute.

The experiment was carried out in a 7500 Fast plate Real-Time PCR system (ThermoFisher Scientific, USA) with a preliminary denaturation phase set for 95°C lasting 15 minutes, followed by 40 rounds comprising denaturation set for 95°C at 15 seconds, and subsequent aligning of primers annealing plus amplification of genetic materials achieved by 60°C temperature maintenance lasting 30 seconds. At amplification phase, fluorescence analyses were obtained. As the experiments get to the exponential phase preceding that of linear phase, the cycle thresholds (C_T) were positioned. Amplification of a 561bp fragment within the 5' ORF encoding non-structural (NS) capsid region was achieved by the ChPV test. The detail of probe as well as primer sequences utilised in the assay is presented (Table 3.9).

Table 3.9: Primers and nucleotide sequences for ChPV qPCR assay

Primer	Nucleotide sequence (5' – 3')
Parvovirus forward	CAT CCC GAA TTT AAC GGG ACT T
Parvovirus reverse	AGG CTG GGC ACA CGG TC
Probe	6FAM-ATT GTG A ACC AGT TCA GCA C-MGB

SECTION FIVE

3.6 DETERMINATION OF PHYLOGENETIC RELATEDNESS BETWEEN DETECTED ENTERIC VIRUSES WITH REFERENCE STRAINS

3.6.1 SEQUENCING OF OBTAINED ENTERIC RNA AND DNA VIRUSES

3.6.1.1 Gel excision and purification

All amplification products at correct range of DNA fragments were cut out from gels. In addition, their extraction was done by means of the PureLink Quick Gel extraction kit (Invitrogen, Life Technologies) according to the specifications of the kit.

3.6.1.1a Gel excision and dissolution

Equilibration of water bath to 50 °C prior to use was carried out. A little part of gel containing the DNA fragment of interest was excised. The excised piece was weighed with a scale having up to 0.001 gram sensitivity and placed in an Eppendorf tube. Buffer for gel dissolution was poured to excised gel at the ratio 3:1. This was subsequently incubated at 50 °C for 10 minutes, upturning at three minutes interval to allow proper dissolution and mix of molten gel. Once there is complete gel dissolution, further five minutes incubation was allowed. Then, the tube was removed from the water bath and equal quantity of isopropanol as dissolved gel mixture was included and properly mixed in order to allow for best possible yields of DNA. This was afterward purified using a centrifuge.

3.6.1.1b Purifying DNA using a centrifuge

To prepare the wash buffer, 64 mL ethanol was added to16 mL wash buffer and properly mixed. Using one column per 400 mg of agarose gel, the molten gel solution was pipette in an extraction column within a collection tube to allow the DNA bind to the extraction column. This was spun for one minute at >12,000 x g and the solution set in the collection tube thrown off; the column was re-placed within the collection tube. Washing of bound DNA to membrane of the column was carried out by pipetting 500 µl ethanol-wash-buffer solution directly on the membrane and the buffer solution removed by

spinning the column for a minute at >12, 000 x g. The solution set in the collection tube was thrown off. To get rid of ethanol, the column was further spun at >12, 000 x g for two minutes and the solution set in the collection tube thrown off. To elude DNA, the column was positioned in an Eppendorf tube and 30 µl elution buffer was pipette targeting middle of the column. This was kept on the bench for a minute. Next, there was centrifugation of the tube for a minute at >12 000 x g. Elute contained the distilled DNA which was then kept pending use at -20 ° C.

3.6.1.2 SEQUENCING OF DNA

Purified amplification products were bi directionally sequenced utilizing same primers as used for virus amplification with the Big Dye® Terminator Cycle Sequencing Kit v3.1 (Invitrogen) in line with the manufacturer's protocol. Since the amplicons were sequenced in both directions two sequencing reaction mixes, each of 20 µl volume, with the same contents but different primers (forward or reverse) were set up in PCR tubes for each sample as follows;

1. BigDye sequencing buffer: 2.0 µl
2. Primer (forward/reverse): 4.0 µl of each (1 pmol/µl)
3. Ready reaction mix (RRM): 4.0 µl
4. DNA template: volume depends on size of band obtained following electrophoresis of purified DNA
5. Sterile distilled water: made up to 20 µl, depending on volume of DNA template

Amplification was done in an automatic thermal cycler using a v3.1 sequencing programme with thermal cycling condition of preliminary denaturation lasting a minute at 96 °C, then, 25 rounds of 96 ° lasting 10 seconds, 50 °C lasting 5 seconds and 60 °C lasting four minutes for denaturation, annealing and expansion, respectively. The Dye Ex 2.0 Spin Kit (Qiagen, UK) for dye terminator removal was utilised to distil the products of the reaction as specified by the manufacturers. The procedure involved gentle vortex of the provided spin column to re-suspend the resin while the column lid was slackened a fraction to circumvent vacuity within the spin column. The bottom end of the spin column was then ripped off and the column placed in two mL collection tube and spun for three minutes at 2,750 rpm. The spin column was moved into a sterile micro centrifuge tube with 20 µl reaction mixture gradually applied on the gel bed in the spin column without

touching the gel or the sides of the tube with the tip of the pipette. This was subsequently spun at 2,750 rpm for three minutes. Then the spin column was taken off the tube and discarded; obtained elutes containing the purified DNAs were dried by incubating at 37 °C overnight. The sequencing of test samples was carried out in a DNA (ABI 3730) Analyzer (Thermo Fisher Scientific).

3.6.2 ANALYSIS OF SEQUENCES

Sequence results obtained were evaluated with the Vector NTI suite (Invitrogen). This programme was employed as well to translate and edit the electropherograms of sequences and rule out nucleotide uncertainty. Subsequently, Basic Local Alignment Search Tool (BLASTN 2.8.1+) programme for nucleotides (Morgulis *et al*., 2008) was used to compare DNA sequences on NCBI online platform to confirm their identity.

3.6.3 PHYLOGENETIC ANALYSIS

The related sequences obtained during BLASTN search were retrieved from NCBI GenBank databases and multiple sequence alignment of the retrieved sequences and nucleotide sequences obtained for each identified enteric virus in this study was carried out using ClustalW programme of Molecular Evolutionary Genetics Analysis (MEGA) X software (Kumar *et al*., 2018). The pair wise distance method using the Maximum Composite Likelihood model (Tamura *et al*., 2004) was utilised for working out evolutionary distances. Then, phylogenetic trees were contrived by means of the Neighbour-joining statistical system (Saitou and Nei, 1987), with 1, 000 bootstrap replications. The clustered associated taxa in the bootstrap analysis were revealed close to the branches and rendered as replicate tree percentages (Felsenstein, 1985).

3.6.4 GENE SEQUENCE

The sequences of the nucleotides of identified enteric viruses in the current study were archived in the databases of the Genbank. The accession numbers granted CAstV Nigerian strains NGR_ch1, NGR_ch2, NGR_ch3, NGR_ch4, NGR_Tk1 and NGR_Tk2, are MK509014, MK509015, MK518374, MK518375, MK509016 and MK509017, respectively. Also, Nigerian strains NGR_ANV_Ch, NGR_ARV_Ch and NGR_ChPV_Ch are MN026335, MN026333 and MN026334 for avian nephritis type 2, avian reovirus and chicken parvovirus, respectively. While FAdV Nigerian strains NGR_FAdV_ch1,

NGR_FAdV_ch2 and NGR_FAdV_ch3 are MK509018, MK509019 and MK509020, respectively.

CHAPTER FOUR

RESULTS AND DISCUSSION

SECTION ONE

4.1 Serodetection of astroviruses by indirect immunofluorescent assay

Of the 261 sera tested, 16 (6.1%) were positive after immunofluorescent staining with CAstV-11672-infected cells showing intracytoplasmic inclusion bodies (Figure 4.1) comprising 13 from broilers and three from turkeys.

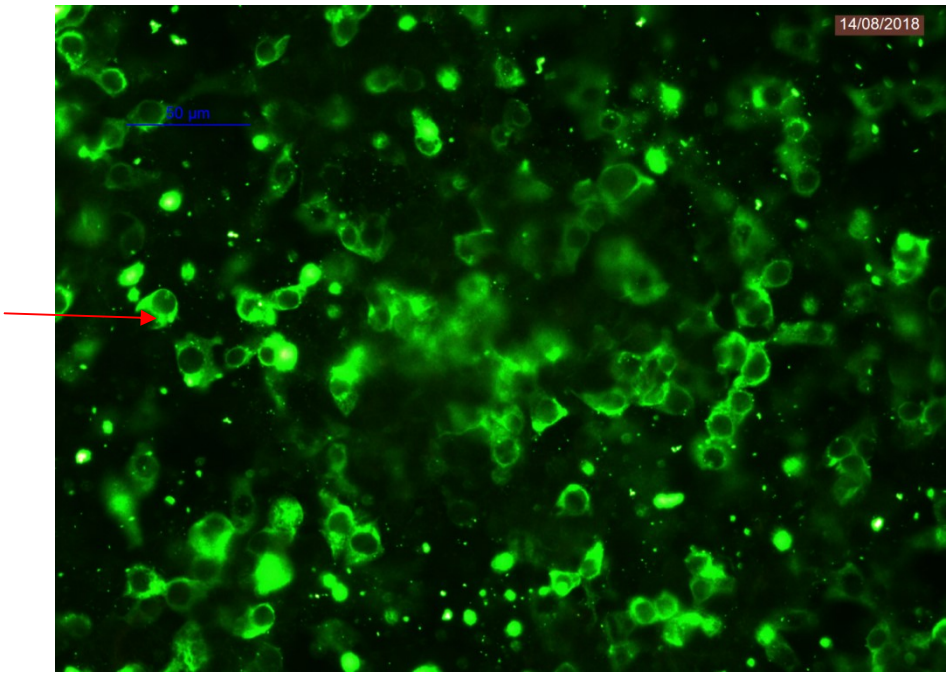


Figure 4.1: Immunofluorescent staining (FITC) with CAstV-11672-infected cells showing intracytoplasmic inclusion bodies (arrow)

Explicitly, out of the six 14-week old broilers, only two were positive while 11 out of the 158 day old chicks showed immunofluorescent staining with CAstV 11672-infected cells showing intracytoplasmic inclusion bodies. Although there were more positive samples in broilers compared to turkeys and more in day-old than older broilers, the differences were not significant ($P>0.05$). On the contrary, all sera tested for CAstV 612 and ANV-1 were negative (Table 4.1).

Table 4.1: Serodetection of avian astroviruses in commercial broiler chickens and turkey poults in Oyo, Ogun and Osun states

		Positive (%)		
	No. tested	CAstV		ANV-1
		612	11672	
Broilers				
Day old	158	0	11 (7.0)	0
14 weeks	6	0	2 (33.3)	0
	164	0	13 (7.9)	0
Turkeys	97	0	3 (3.1)	0
Total	261	0	16 (6.1)	0

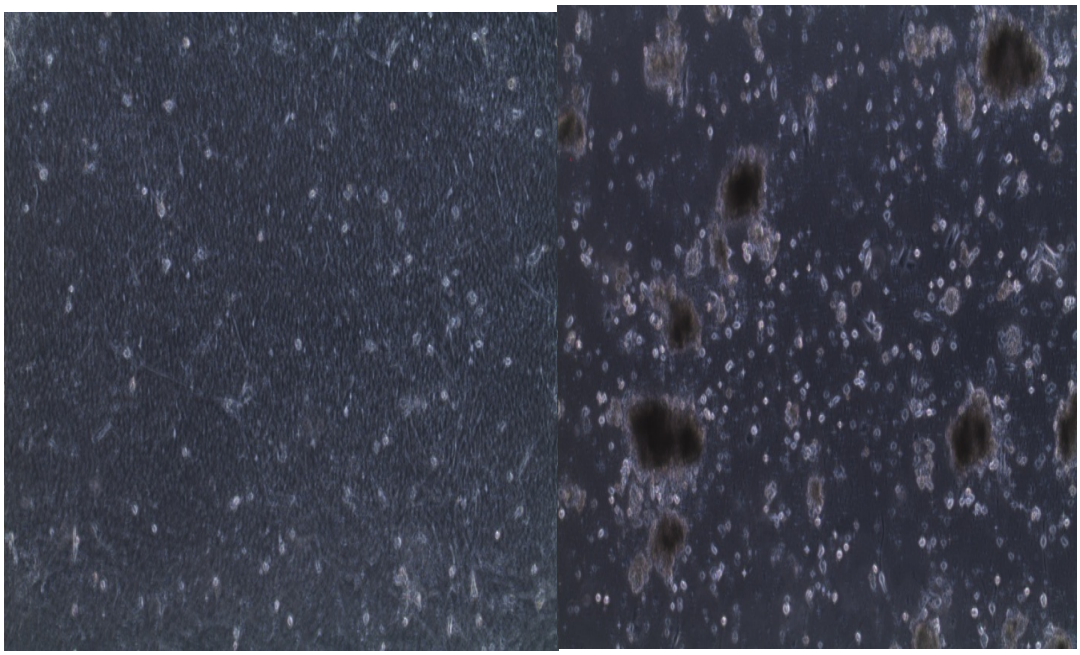
4.2 SECTION TWO

4.2.1 Isolation of enteric viruses in Chicken Embryo Liver Cells

Four of the 261 infected chicken embryo liver cell cultures showed cytopathic effects (Figure 4.2) after 72 hours incubation. These four samples were all from broiler chickens comprised of three and one, respectively from day old and 14-week old broilers.

4.2.2 Identification of enteric viruses using Transmission Electron Microscopy

With the negative-stained samples viewed by electron microscopy at x50, 000 magnification adenovirus-like particles were observed (Figure 4.3) in three of the four isolates with diameter over 50 nm and having hexagonal outline. All the three samples were from day-old broilers (Table 4.2). Furthermore, pleomorphic virus-like particles were seen (Figure 4.4) with average dimension of 170 nm varying between 168 - 174 nm. They appear tasseled (fimbriated) showing exterior protrusions similar to viruses within Paramyxoviridae unit.



A **B**

Figure 4.2: Chicken Embryo Liver cells with (A) uninfected cells (control). Infected cells with presentation of cell sloughing as well as death (B)

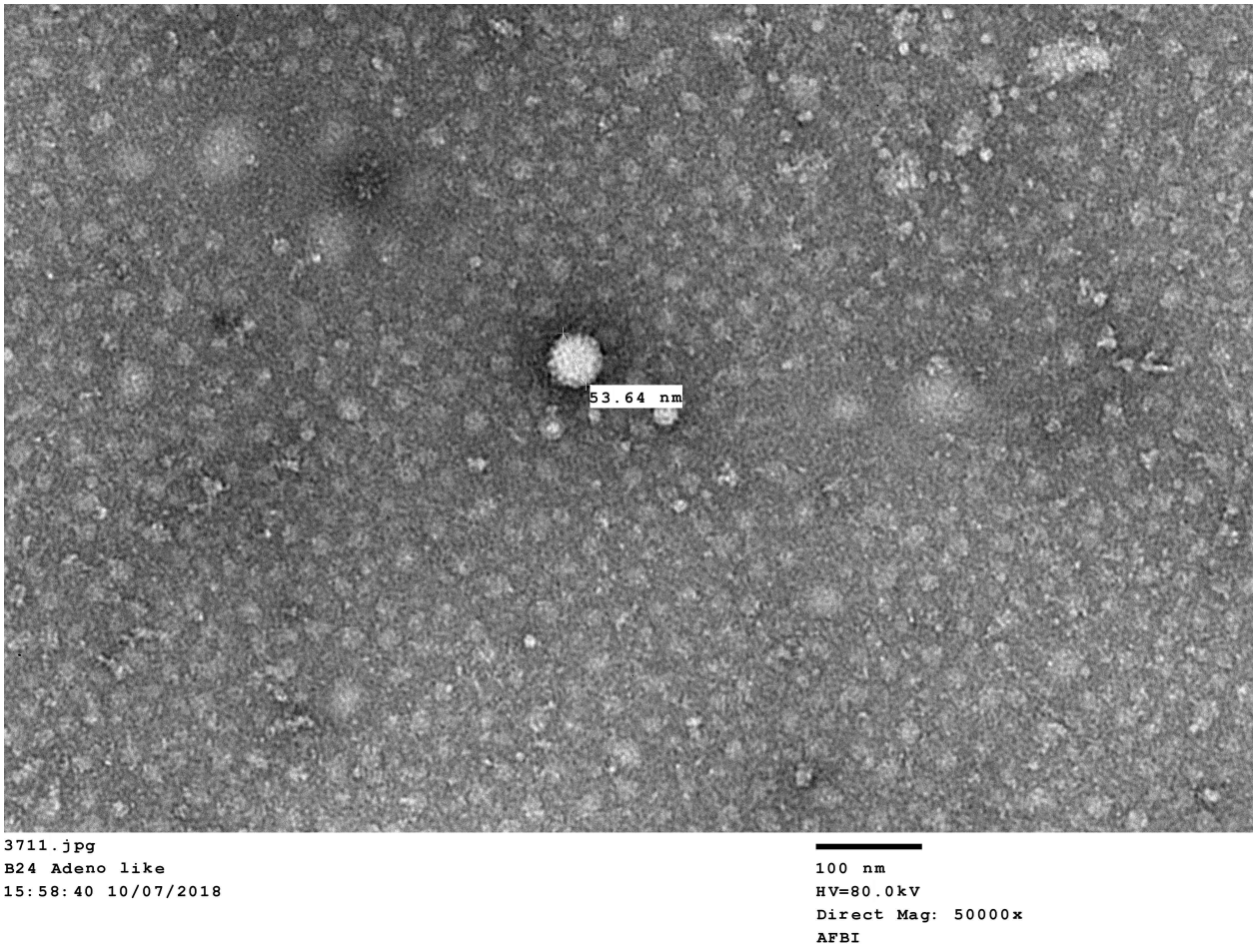


Figure 4.3: Adenovirus-like particle visualized with transmission electron microscopy using negative staining

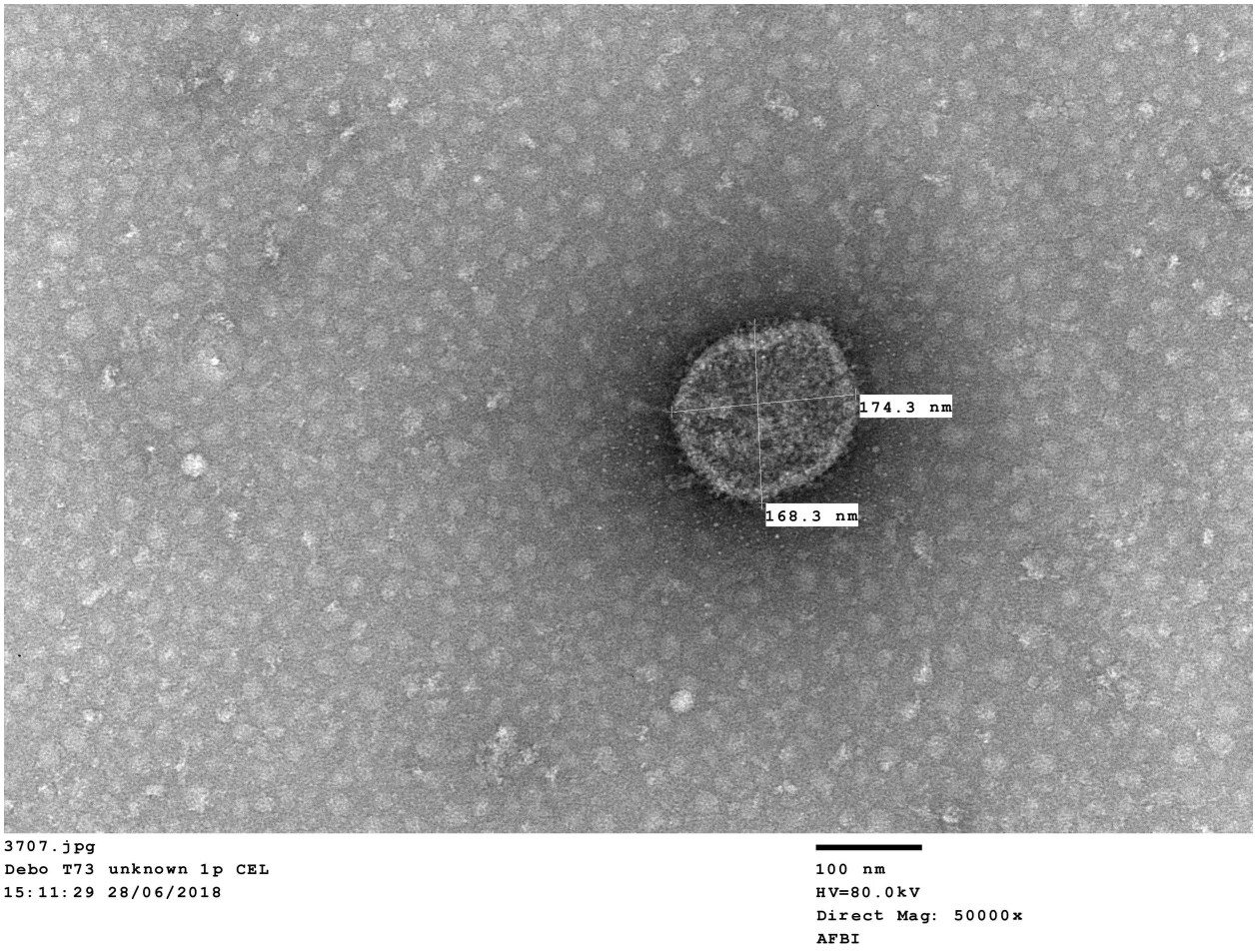


Figure 4.4: Unknown virus-like particle visualized with transmission electron microscopy using negative staining

Table 4.2: Detection of fowl adenovirus in cell culture isolates by transmission electron microscopy

Sample	VI passage level	EM result
1	1p	+
2	3p	+
3	1p	-
4	1p	+

Passage (p), positive (+), negative (-)

SECTION THREE

4.3 Detection of enteric viruses using conventional RT-PCR and PCR

4.3.1 CAstV and ANV capsid protein RT-PCR assays

With the capsid RT-PCR assays, a fragment of approximately 2.2 kb capsid gene (ORF2) product from CAstVs and ANVs was amplified (Figure 4.5). For CAstV, 133 out of the tested chicken samples produced bands at the expected size of 2.2 kb while 17 out of the tested turkey samples showed expected band size (Table 4.2). There was a significant difference ($P<0.05$) in CAstV RNA detection in broilers and turkeys with odd ratio of 20.2 (95%CI: 10.5-37.3). For ANV, only one out of the tested samples from chickens gave the expected band size while all samples from turkeys were negative for ANV.

4.3.2 Avian rotavirus RT-PCR

Assay to amplify the non-structural protein 4 (NSP4) of group A avian rotaviruses (AvRV) in all the tested samples from commercial broilers and turkeys were negative as they did not produce bands at the expected size of 630 bp.

4.3.3 Avian reovirus RT-PCR

Conventional RT-PCR carried out for ARV using primers that amplified fraction of the sigma C protein (Kant *et al*., 2003) predetermined by the ORF of the S1 fragment revealed expected band size of 1.1 kb (Figure 4.6) in six samples from commercial chickens.

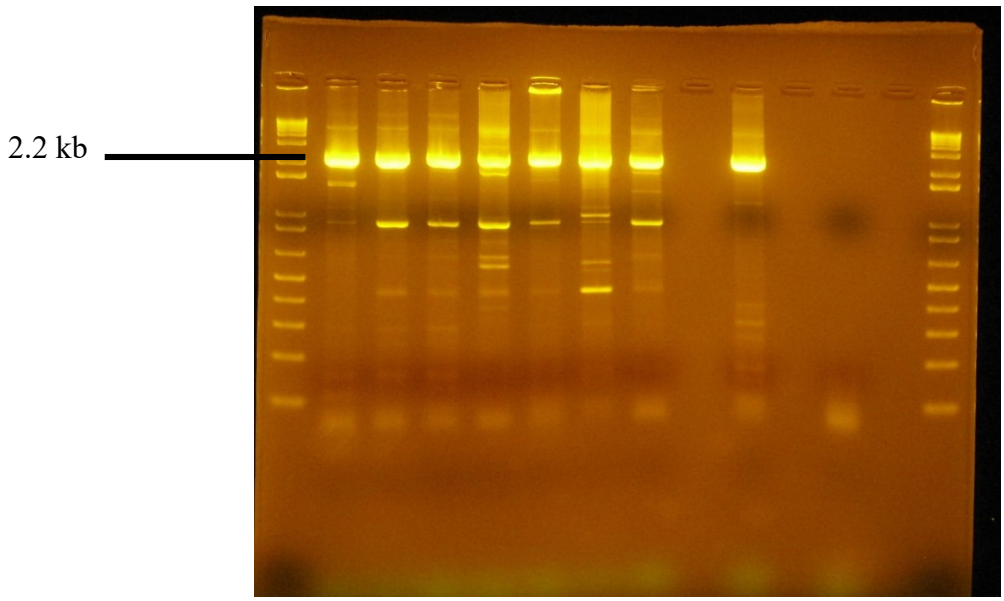


Figure 4.5: Capsid gene RT-PCR assays for CAstV and ANV detection in commercial broiler chickens and poults

Legend

L: Molecular weight markers (1 kilo base plus), 1-7: test samples, P: positive control, N: negative control

Table 4.3: Capsid RT-PCR assays for CAstV and ANV detection in commercial broiler chickens and poults

	No. tested	No. positive (%)	
		CAstV	ANV
Chicken	164	133* (81.1)	3 (1.8)
Turkey	97	17 (17.5)	0
Total	261	150 (57.5)	3 (1.1)

*Significant difference ($P<0.05$)

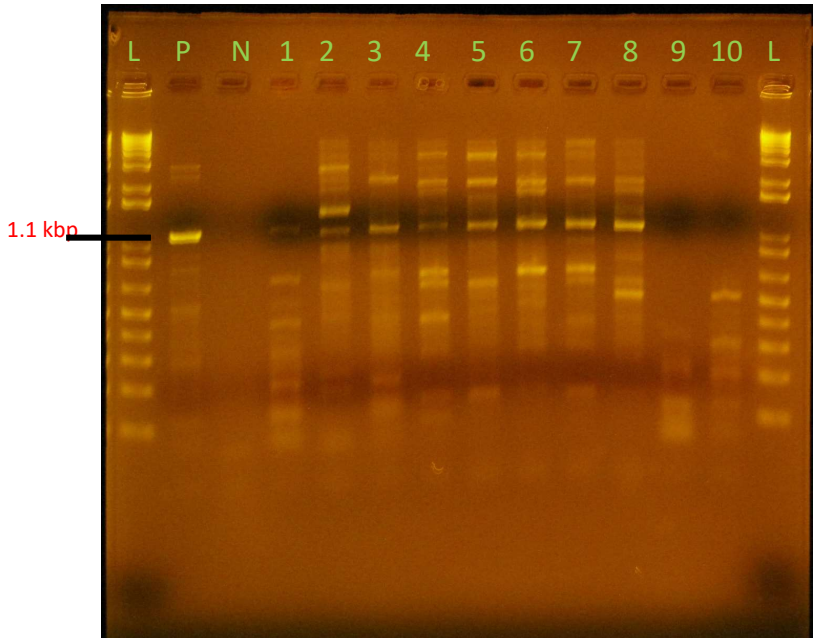


Figure 4.6: RT-PCR detection of avian reovirus (ARV) in commercial broilers

Legend

L: Molecular weight markers (1 kilo base plus), P: positive control, N: negative control, 1-10: test samples

4.3.4 Turkey astroviruses 1 and 2 RT-PCR

Assay to amplify the polymerase gene of turkey astroviruses -1 and -2 in all the tested samples from commercial broilers and turkeys were negative as none produced bands at the expected sizes of 251 bp and 911 bp for turkey astroviruses 1 and 2, respectively.

4.3.5 Parvovirus non-structural protein gene PCR

The conventional PCR carried out for parvoviruses using primer set described by Zsak *et al*. (2008) amplified the NS gene at expected band size of 561 bp (Figure 5.7) in six samples from 14-week-old broilers.

4.3.6 Fowl adenovirus hexon gene PCR

The conventional PCR carried out for FAdV using specified primer set amplified the hexon gene of adenovirus (Meulemans *et al*., 2001) at expected band size of 900 bp (Figure 4.8) in three samples from day old chicks.

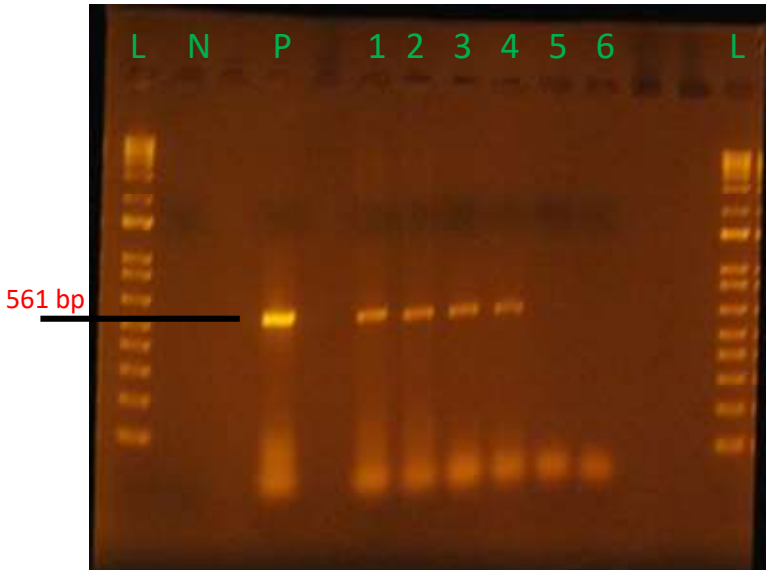


Figure 4.7: PCR detection of chicken parvovirus (ChPV) in commercial broilers

Legend

L: Molecular weight markers (1 kilo base plus), 1-6: test samples, P: positive control, N: negative control

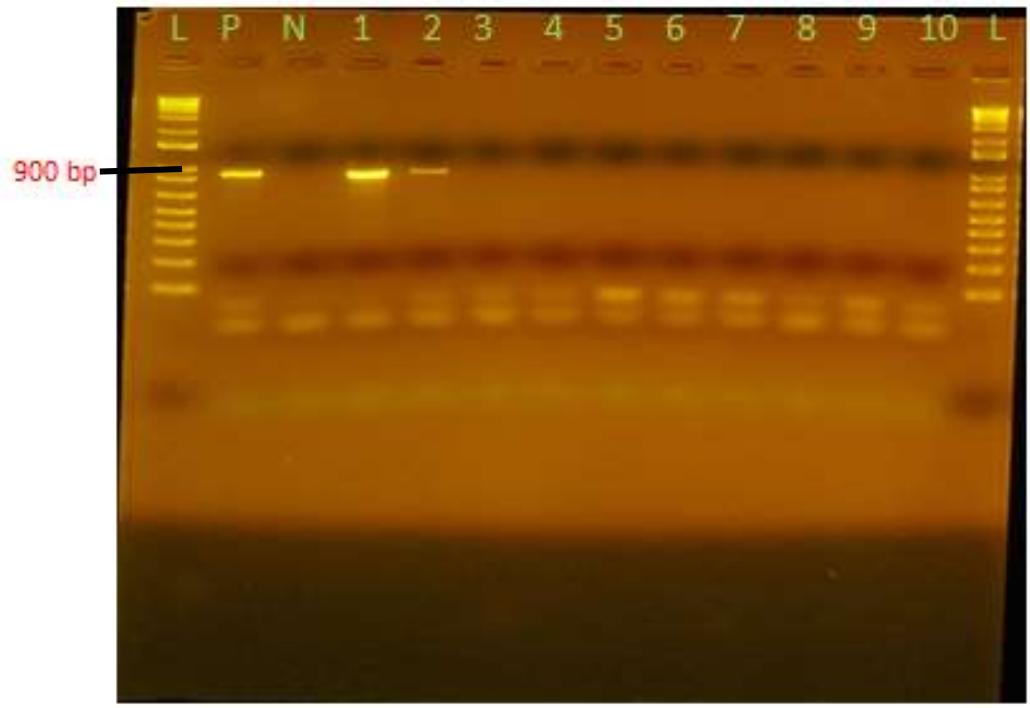


Figure 4.8: PCR detection of fowl adenovirus (FAdV) in commercial broilers

Legend

L; Molecular weight markers (1 kilo base plus), P; positive control, N; negative control, 1 to10; test samples

SECTION FOUR

4.4 Quantification of CAstV, ANV and ChPV

4.4.1 CAstV and ANV RT-qPCR

The real time RT-PCR for CAstV detection gave an amplification plot showing cycle thresholds (Figure 4.9). Quantification with real-time RT-PCR detected CAstV RNA (log values 2.2-8.0) in 100% (164/164) of chickens tested with most of the samples (63.4%) having high (>6.0) RNA levels (Table 4.4). In turkeys, the real-time assays detected CAstV RNA (log values 2.3- 6.2) in 83.5% (81/97) of the samples tested while 10.5% (16/97) were negative (Table 4.5).

The real-time RT-PCR for ANV detection gave an amplification plot showing cycle thresholds (Figure 4.10). For ANV, RNA (2.8-4.9) was detected in 4.9% (8/164) with most having low (2.0-4.0) RNA levels (Table 4.6) while ANV was not detected in turkeys.

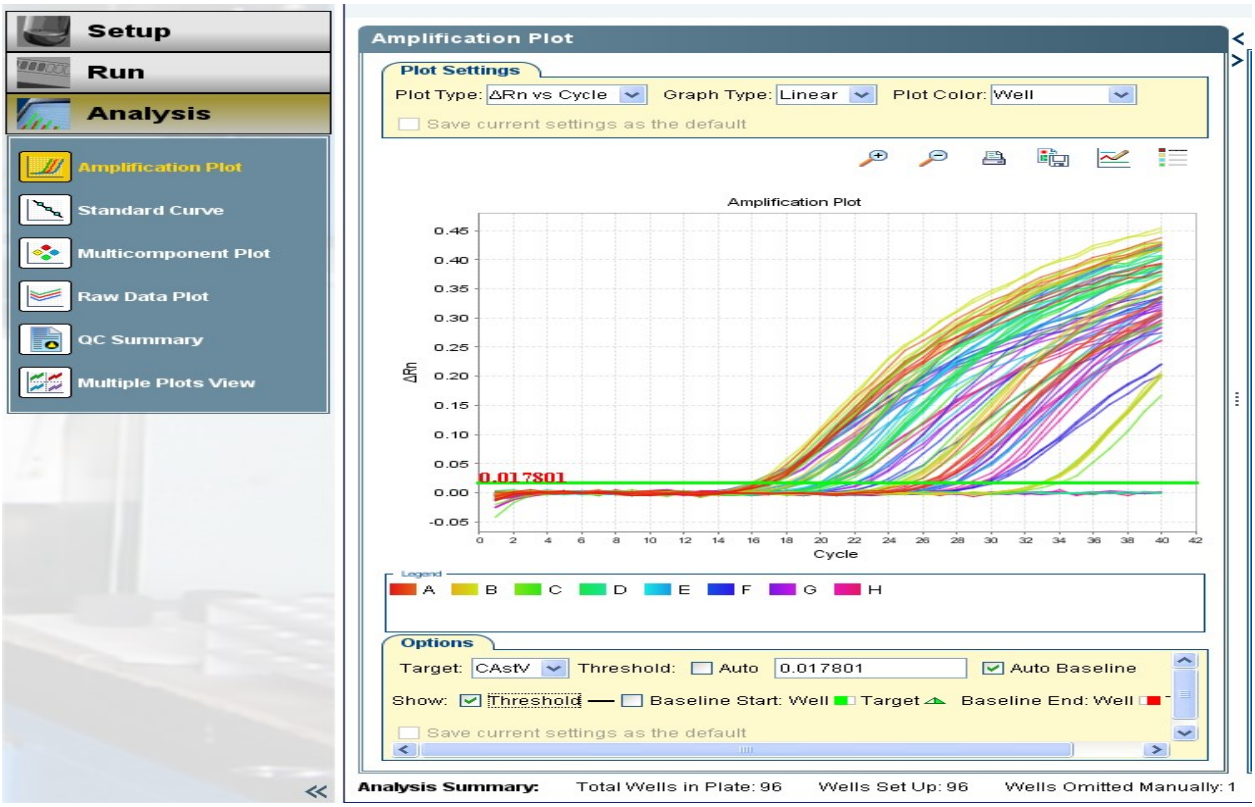


Figure 4.9: Real-time amplification of CAstV RNA in commercial broilers and turkeys

Table 4.4: Evaluation of CAstV RNA by Real time RT-PCR in commercial broilers

RNA levels (Log. Value in base 10)	Number of samples	Percentage
High (>6.0)	104	63.4
Intermediate (4.1-6.0)	45	27.4
Low (2.0-4.0)	15	9.1
Total	164	100.0

Table 4.5: Evaluation of CAstV RNA by Real time RT-PCR in commercial turkeys

RNA levels (Log. Value in base 10)	Number of samples	Percentage
High (>6.0)	5	5.2
Intermediate (4.1-6.0)	36	37.1
Low (2.0-4.0)	40	41.2
Total	81	83.5

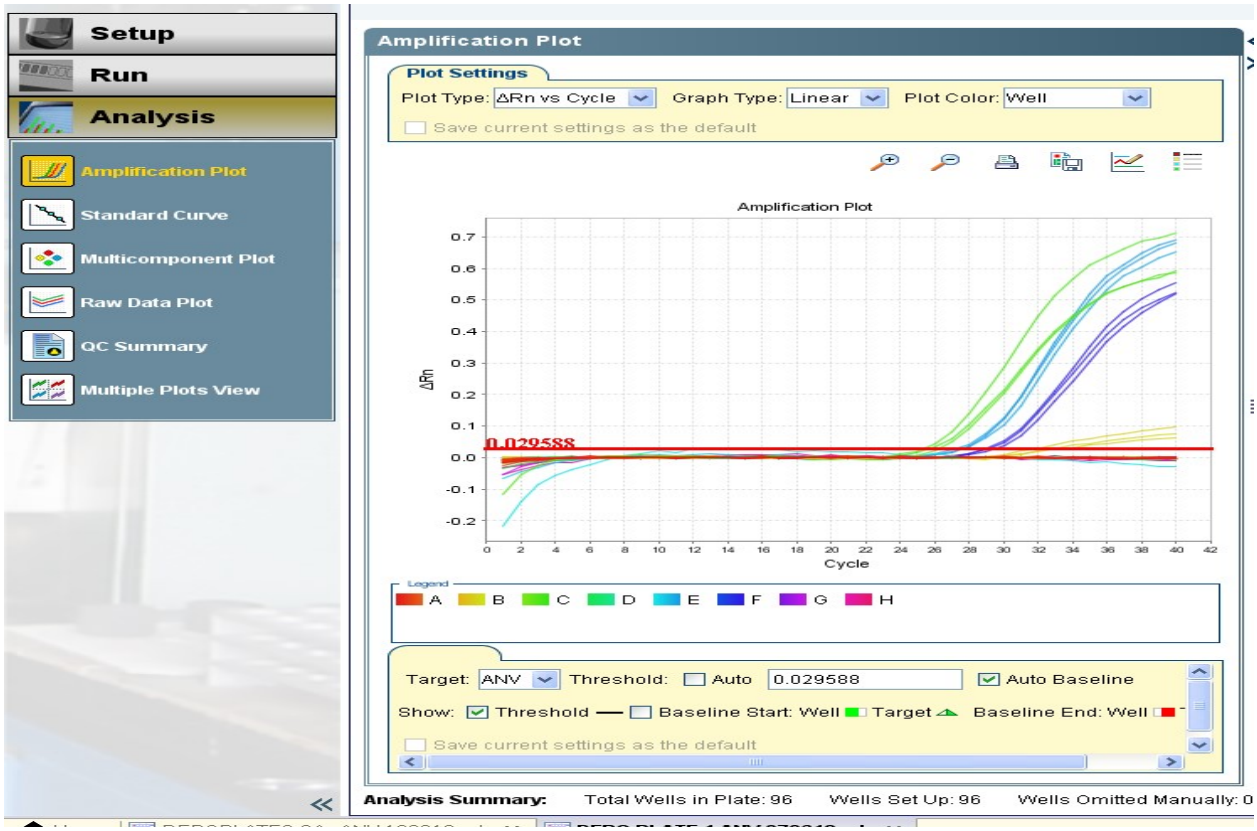


Figure 4.10: Real-time amplification of ANV RNA in commercial broilers

Table 4.6: Evaluation of ANV RNA by Real time RT-PCR in commercial broilers

RNA levels (Log. Value in base 10)	Number of samples	Percentage
High (>6.0)	0	0
Intermediate (4.1-6.0)	3	1.8
Low (2.0-4.0)	5	3.0
Total	8	4.9

4.4.2 ChPV qPCR

The real-time PCR detected ChPV DNA in 6 (3.7%) samples from the older broilers (Table 4.7), while all the day-old broilers and turkeys were negative.

Table 4.7: Evaluation of ChPV DNA by Real time PCR in commercial broilers

DNA levels (Log. Value in base 10)	Number of samples	Percentage
High (>6.0)	0	0
Medium (4.1-6.0)	3	1.8
Low (2.0-4.0)	4	2.4
Total	7	4.3

SECTION FIVE

4.5 DETERMINATION OF PHYLOGENETIC RELATEDNESS BETWEEN DETECTED ENTERIC VIRUSES WITH REFERENCE STRAINS

4.5.1 Nucleotide sequence alignment

Target genes of the identified enteric viruses were all successfully amplified. Alignment of CAstV nucleotide sequences disclosed high similarity with 98-100% homology to group Bi of CAstVs by the capsid protein gene analysis. The ARV identified showed 83% similarity by the σ C protein to strain Gel13b98 which is classified in cluster 3, from a case of malabsorption/tenosynovitis from Germany in 1998 (Kant *et al*., 2003) and the ChPV identified showed 96% similarity with enteric chicken parvovirus strain ChPV/G090 from Polish commercial poultry flocks (Domanska-Blicharz *et al*., 2012). With the analysis of hexon protein gene sequence and in line with the International Committee on Taxonomy of Viruses (ICTV) nomenclature system, out of the three identified FAdVs, two were typed as ICTV serotype 4, species C and one as serotype 5, species B. The ANV showed 80% homology to ANV-2 in chickens from Japan and United Kingdom by the ORF2 (capsid) gene (Imada *et al*., 2000; Todd *et al*., 2011). Details of the utilised reference sequences from NCBI are shown (Table 5.14a-e)

Table 4.8a: CAstV capsid sequences for phylogenetic investigation

	Reference	Locality	Accession Number
1	CAstV WCS	Canada	KY635970.1
2	VF11-66B WCS	Finland	MY482110.1
3	CAstV/01/17/HR	India	MF405736.1
4	PRDC/576 north zone	India	JX945883.1
5	PRDC/533; south zone	India	JX945859.1
6	PRDC/574; north zone	India	JX945862.1
7	Astrovirus isolate 301-4	Italy	JQ307839.1
8	CAstV 11672 Bi	United kingdom	JN582327.1
9	VF06-1/4	United kingdom	JN582309.1
10	CAstV 11522	United states	JN582305.1
11	VF06-7/5	United kingdom	JN582310.1

Table 4.8b: ChPV sequences for phylogenetic investigation

	Reference	Locality	Accession number
1	Chicken parvovirus Strain ChPV/ Poland/ G090	Poland	JQ178302.1
2	Chicken parvovirus isolate CAN-5 ChPV	Canada	JF267314.1
3	Turkey parvovirus strain TuPV/Poland/G048	Poland	JQ178321.1
4	Turkey parvovirus (Tu1/VA/00) strain	USA	JX207118.1
5	Turkey parvovirus strain TuPV/Poland/G006	Poland	J178317.1
6	Chicken parvovirus (USP 238-1) isolate	Brazil	MH176307.1
7	Chicken parvovirus-CAN-41	Canada	JF267318.1
8	Chicken parvovirus Ch1515/2007/HUN	Hungary	HM208288.1
9	Turkey parvovirus isolate CRO-844	Croatia	JX114938.1
10	Turkey parvovirus (Tu3/PA/09) strain	USA	JX207131.1
11	Turkey parvovirus isolate CRO-876	Croatia	JX114940.1
12	Chicken parvovirus isolate CAN-50	Canada	JF267322.1
13	Turkey parvovirus Tu762/2009/HUN	Hungary	HM208287.1
14	Turkey parvovirus (TuPV/LT521) isolate	USA	KU569262.1

Table 4.8c: ARV sequences for phylogenetic investigation

	Reference	Locality	Accession Numbers
1	NS Strain-V-06 protein gene	Hungary	KX398238.1
2	16821-M-06 NS protein gene	Hungary	KX398308.1
3	Isolate-16-0753A/16 Sigma C (S1) gene	Canada	MG822677.1
4	Isolate-16-0753B/16 Sigma C (S1) gene	Canada	MG822676.1
5	Isolate-16-0711/16 Sigma C (S1) gene	Canada	MG822679.1
6	Strain T1781 Segment S1	Hungary	KC865792.1
7	Isolate-07634/14 Sigma C gene	USA	KR856992.1
8	Isolate-100192 S1 (Sigma C) gene	USA	KJ879700.1
9	Isolate 99848 S1 (Sigma C) gene	USA	KJ879690.1
10	Isolate-99847 S1 (Sigma C) gene	USA	KJ879689.1
11	Isolate 97362 S1 (Sigma C) gene	USA	KJ879648.1
12	Isolate-99477 S1 (Sigma C) gene	USA	KJ879653.1
13	Isolate-95403 S1 (Sigma C) gene	USA	KJ803969.1
14	Isolate-22790/11 S1 (Sigma C) gene	USA	KP727787.1
15	Isolate-03422/14 S1 (Sigma C) gene	USA	KP727788.1

Table 4.8d: FAdV sequences for phylogenetic investigation

	Reference	Locality	Accession number
1	FAdV_HR2_FAdV8_Mittal	India	HM748589
2	FAdV_JSJ13_Zhao	China	KM096544
3	FAdV4_ATCCVR829_J2A	Belgium	AF339917
4	FAdV4_KR5_hexon gene	Belgium	AF508951
5	FAdV5_340	Australia	EU979371
6	FAdV5_340_ hexon gene	Belgium	AF508952
7	FAdV3_ATCC_VR828_IBH2A_ hexon gene	Belgium	AF339916
8	FAdV9_ strain A02	Australia	EU979376
9	FAdV9_ATCC_VR833_A2A	Belgium	AF339918
10	FAdV3_SR49	Australia	EU979369
11	FAdV_D_57502	Canada	EF685529
12	FAdV2_Kajan2013	Hungary	KC750793_IBH
13	FAdV_D_1340_11	Sweden	JX25176
14	UK09/97190_Marek2010	United Kingdom	FN869963

Table 4.8e: ANV sequences for phylogenetic investigation

	Reference	Locality	Accession Numbers
1	ANV isolate -SEP (AN-368-2005)	United States of America	HQ188694.1
2	ANV isolate 3	Iran	KC811068.1
3	ANV isolate -SEP AN-458-2005	United States of America	HQ1880699.1
4	ANV isolate DE-CK-811-2005	United States of America	HQ1880693.1
5	ANV strain 45-4	Brazil	KU711059.1
6	ANV strain 46-1	Brazil	KU711065.1
7	ANV strain 46-4	Brazil	KU711064.1
8	ANV strain 46-2	Brazil	KU711063.1

4.5.2 Phylogenetic analysis

A phylogenetic tree derived from the analysis of the multiple alignment of capsid gene fragment nucleotide sequences of the Nigerian CAstV strains and other reference strains showed six clusters with the Nigerian strains found in two of them (Figure 4.11).

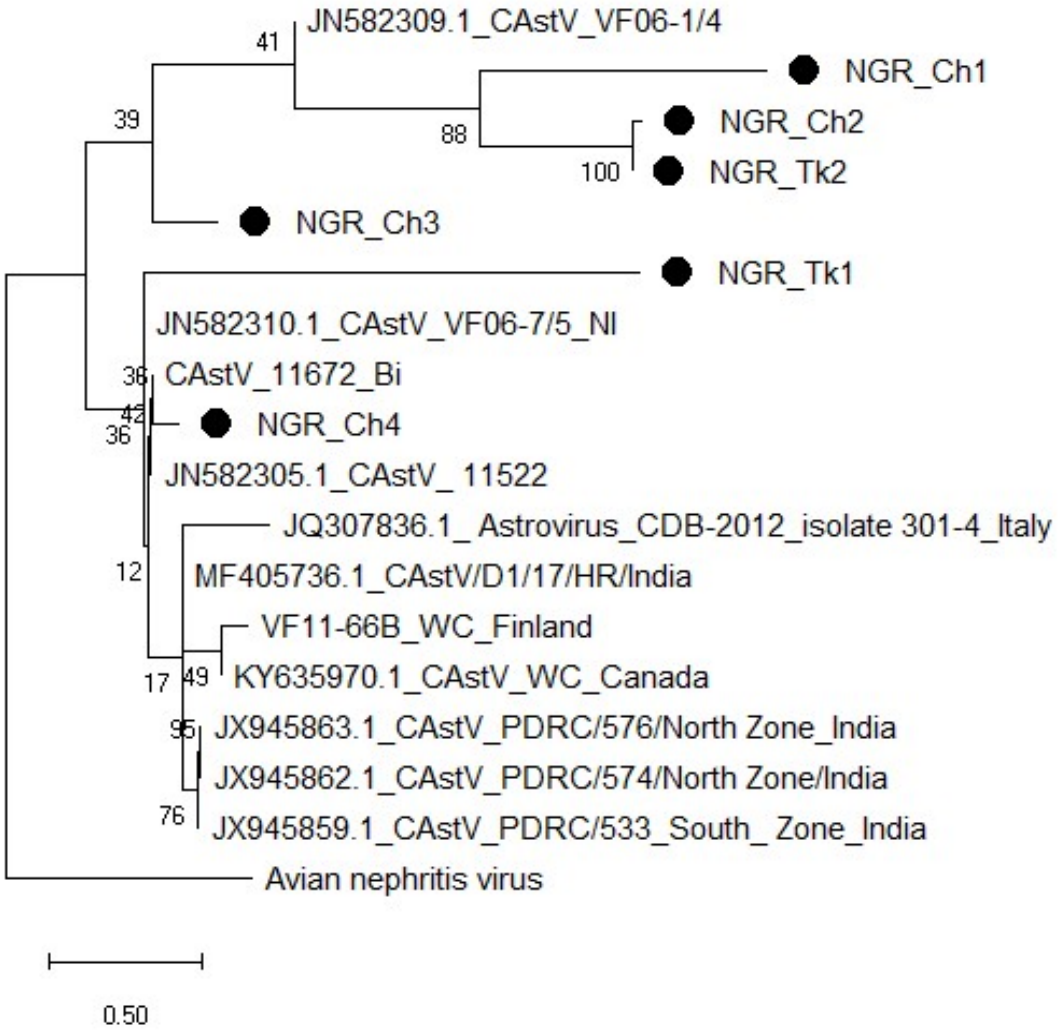


Figure 4.11: Phylogenetic relationship among Nigerian (indicated by black dot) and reference CAstV strains established by sequences of the capsid protein nucleotides

Phylogenetic tree derived from the σ C gene fragment nucleotide sequences analysis of the Nigerian ARV strains and other NCBI reference strains showed unique cluster of the Nigerian strains (Figure 4.12).

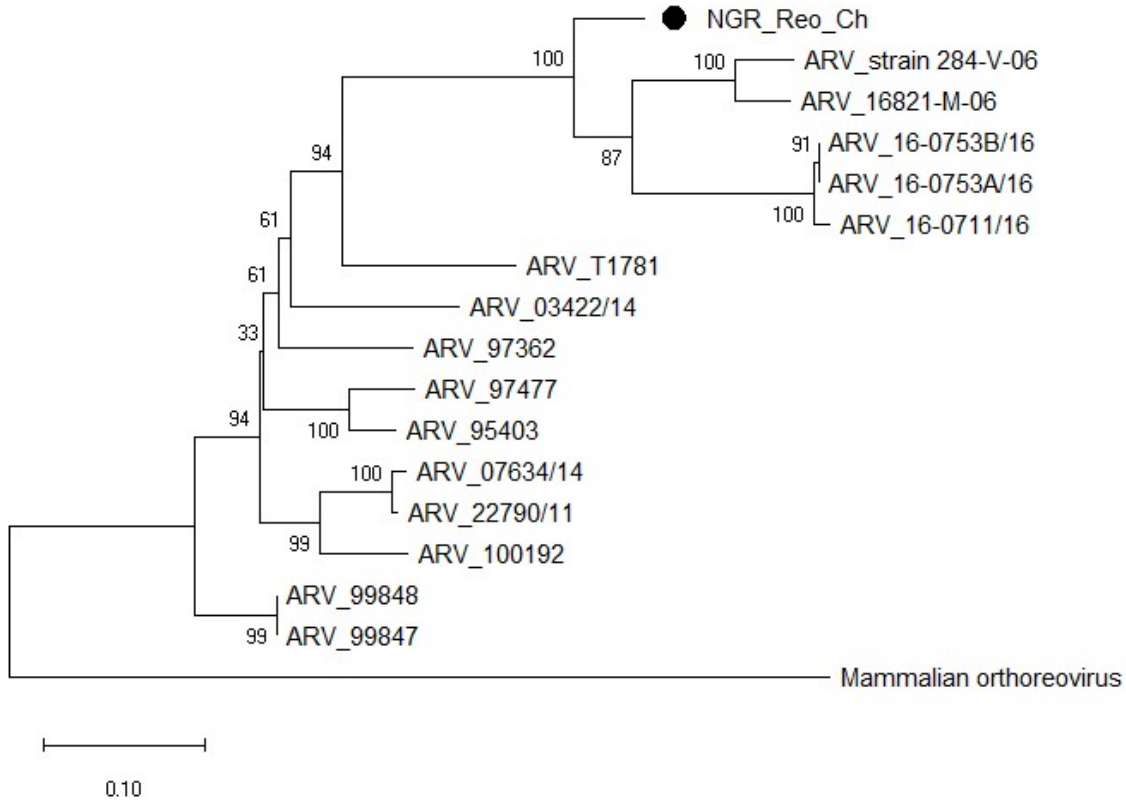


Figure 4.12: Phylogenetic relationship among Nigerian and reference strains of ARV established by nucleotide sequence of σ C protein

A phylogenetic tree derived from the alignment evaluation of NSP gene fragment nucleotide sequences of the Nigerian ChPV strain and other NCBI reference strains of parvoviruses showed unique cluster of the Nigerian strain (Figure 4.13).

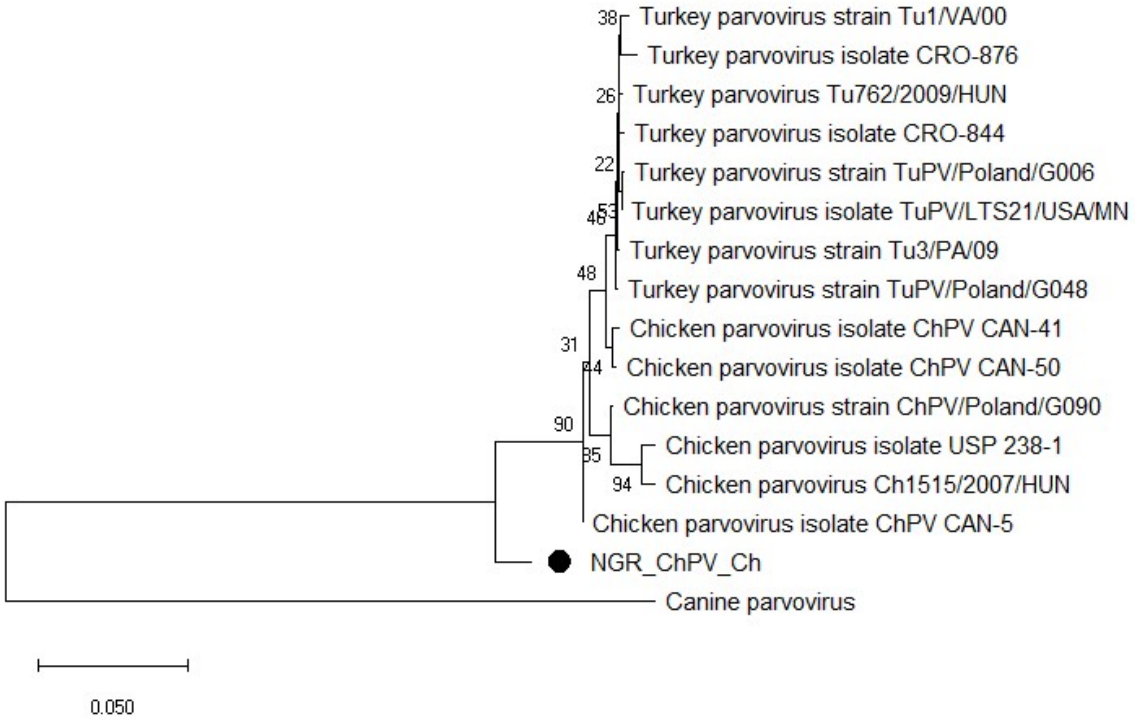


Figure 4.13: Phylogenetic relationship among Nigerian and reference ChPV strains established by sequences of the non-structural protein nucleotides

A phylogenetic tree derived by the alignment of the hexon gene fragment of the Nigerian FAdV strains and other NCBI reference strains sequences showed unique cluster of the Nigerian strain. NGR_ch1 and NGR_ch3 clustered with serotype 4 strains while NGR_ch2 clustered with serotype 5 strains (Figure 4.14).

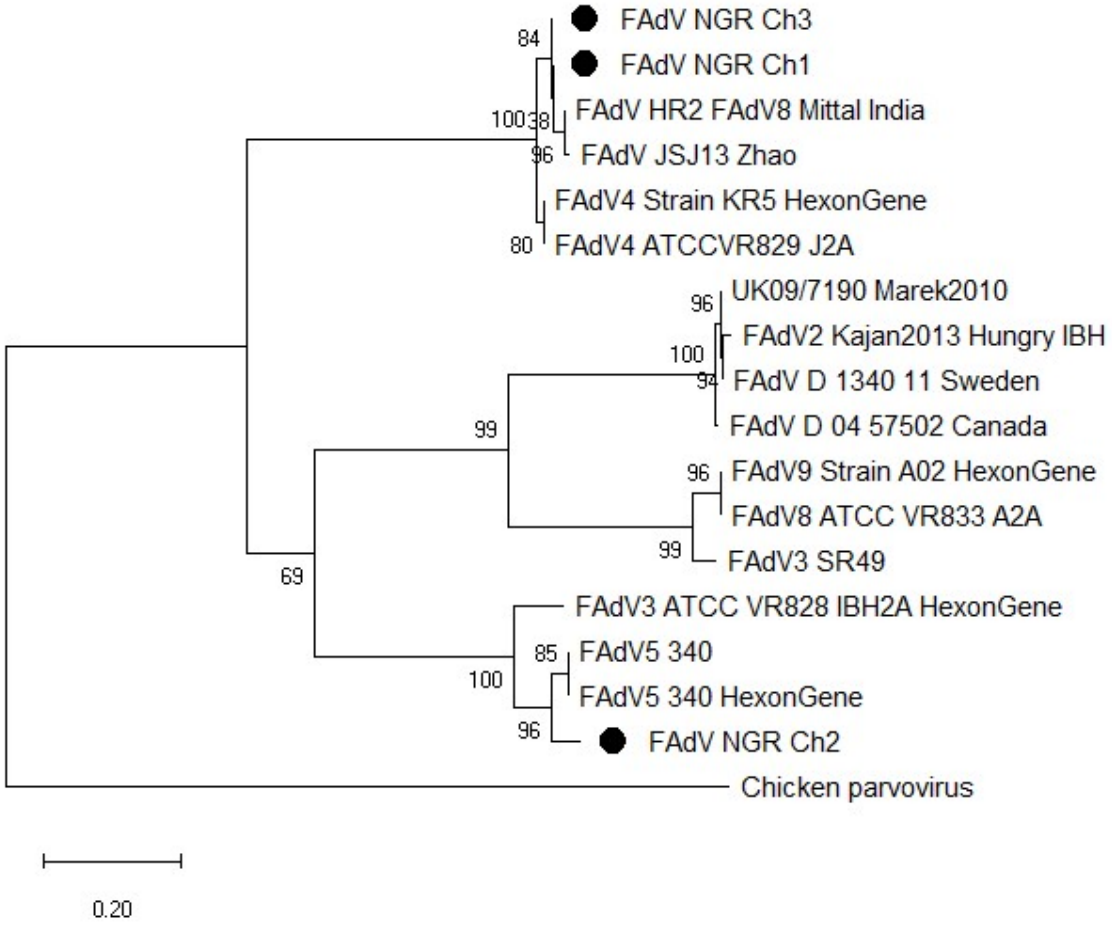


Figure 4.14: Phylogenetic relationship among Nigerian and reference FAdV strains according to hexon gene sequence analysis

Phylogenetic tree obtained from capsid protein gene fragment comparisons of nucleotide sequences of the Nigerian ANV strain and other NCBI reference strains showed unique cluster of the Nigerian strain (Figure 4.15).

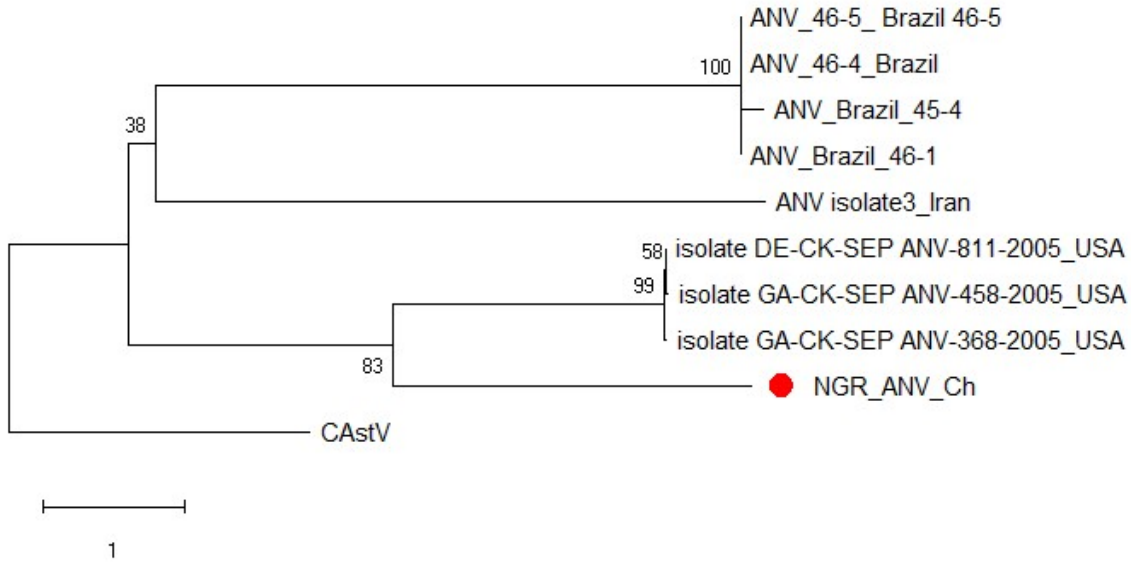


Figure 4.15: Phylogenetic relationship among Nigerian and reference ANV strains based on nucleotide sequence of capsid protein gene

4.6 Discussion

Globally, runting-stunting syndrome (RSS) is a production dilemma distressing poultry with consequential economic non-viability owing to poor feed adaptation, cull increase as well as size irregularity at harvest affiliated with increased expenses on prophylaxis (Long *et al*., 2017). This disease condition plays a significant role in the physical condition of poultry gut as well as susceptibility to diseases with avastroviruses being commonly incriminated. Although enteric infections of birds may be established immediately after hatch with presentations of poor growth and abnormal feathering, infection may persist right through all ages of birds with or without observable clinical signs (Nunez *et al*., 2016). Additionally, infections in birds are commonly linked to a wide range of different strains of chicken and turkey avastroviruses. Therefore, analytical tests should be able to identify and differentiate among diverse genotypes. In this study, using IIF test, CAstV-11672 antibodies were detected in commercial broiler chickens and turkeys in south west Nigeria while CAstV 612 and ANV-1 were not detected. Previous studies revealed a widespread of CAstV antibodies in broiler and different categories of breeder flocks in different continents of the world (Todd *et al*., 2009; Smyth, 2017).

In view of the fact that CAstV vaccine is presently unavailable, the detection of antibodies to CAstV in commercial broilers and poults, although at low prevalence (7.9% and 3.1% correspondingly), signifies a natural exposure of the birds to the virus. This finding substantiates the observation of low prevalence of CAstV antibodies in chickens in south west Nigeria (Oluwayelu and Todd, 2012). However, while the earlier study reported antibodies against CAstV 612 and 11672 in adult chickens, this study only detected antibodies against CAstV 11672 in day old and 14-week old birds. This difference may be due to the fact that this study involved commercial broiler chickens and turkey poults with runting-stunting syndrome as against local chickens that were apparently healthy in the earlier study. Also, the dissimilarity could be as a result of difference in age of studied birds. More so, the detection of CAstV antibodies in turkey samples gives serological indication that turkeys are CAstV -infected or by antigenically related viruses with CAstVs (Todd *et al*., 2009) as well as suggests cross-species transmission in the study area. This is supported by similar finding of CAstV in turkeys (Awe *et al*., 2015).

Additionally, low cross reactivity of CAstV within groups A and B have been reported (Todd *et al*., 2009) and considering CAstV 11672 as a prototype group B CAstV, the detection of antibodies against precise CAstV (CAstV 11672) in day-old broiler chickens and turkeys with RSS indicates vertical transmission of CAstV 11672 or CAstV that is antigenically similar to the CAstV 11672 isolate in poultry. This vertical transmission of CAstV may possibly be from hen to progeny through the egg. Also, it could be set down from cloaca of the hen or litter within nest enclosure which may infect the embryo through shell infiltration and infect the developing embryo. In addition, the detection of CAstV antibodies in older (14-week-old) broiler birds may suggest unresolved persistent infection. The resistance to commonly used disinfectants by this virus (Todd *et al*., 2009) may have played a role in this possible disease sustenance in these birds. Thus, these findings show that exposure to CAstV infection can take place as early as few days (Todd *et al*., 2009; Smyth, 2017) after hatch and beyond.

The occurrence of virus antibodies specifically against chicken astrovirus in the present study implies CAstV 11672 strain or antigenically similar strain circulation in commercial broilers and turkeys with runting-stunting syndrome in the study area. However, there is a need to exercise caution in explanation of the outcomes not positive, considering the growing diversity of CAstV in commercial poultry (Todd *et al*., 2009). Therefore, there is a need for more sensitive and specific detection approaches. In the same manner, bearing in mind the involvement of other enteric viruses in RSS in poultry and their need for growth enrichment, virus propagation in cell culture may be imperative.

In this study, four out of the 261 intestinal content suspensions inoculated into chicken embryo liver cell cultures (CELC) showed cytopathic effect of cell sloughing and death. Furthermore, adenovirus particles were identified in three out of the four negative-stained samples through electron microscopy. The identification of aviadenovirus particles in day old broilers with RSS supports fowl adenovirus involvement in chickens as well as indicates possible vertical transmission of aviadenoviruses in these birds in the study area. Aviadenoviruses have been associated in poultry with RSS and other conditions elsewhere (Koo *et al*., 2013). On the other hand, aviadenoviruses can become opportunistic pathogens especially in case of concurrent infections, severely affecting the health of affected birds (Koo et al., 2013). Therefore, there is a need to characterise and confirm the

identified aviadenoviruses by more sensitive and specific method such as molecular detection by polymerase chain reaction and nucleotide sequencing.

In addition, electron microscopic images of two negatively stained samples revealed pleomorphic virus-like particles appearing fringed or fimbriated with surface projections resembling family members within Paramyxoviridae. Similar particles have been accounted for in faeces of diseased game birds presenting symptoms of stunting and scour (Gough *et al*, 1985) where it was speculated that they may be perhaps explicit for a precise disease state and of analytical significance. Therefore, the characteristic unique morphology of these virus-like fimbriated particles observed in day old commercial broilers with runting-stunting syndrome is suggestive of a hitherto uncharacterised virus needed to be further confirmed.

Cell culture and negative stain electron microscopy have facilitated the virus isolation and identification of adenovirus and pleomorphic virus-like particles appearing fringed or fimbriated with surface projections resembling members of the Paramyxoviridae family in samples from day old commercial broiler chickens with runting-stunting syndrome in this study. However, some enteric viral pathogens are not easily isolated because of their poor cultivation in cell cultures and also, some viruses may lose identifying projections and may be too widely spaced on the grids or among debris to make positive identification complicated by negative stain electron microscopy. Hence, it is imperative to utilise more sensitive and specific detection techniques such as molecular identification of enteric viruses that relies on recognition of deoxyribonucleic or ribonucleic acids unique exclusively for such pathogens and nucleotide sequencing to characterise the identified pathogens.

Despite studies on enteric viruses in poultry for decades, there is still a great deal to find out about poultry enteric diseases. Numerous studies worldwide have exposed the widespread episodes of enteric viruses in poultry flocks with poor growth performance, enteritis and hatchery diseases (Sajewicz-Krukowska *et al.*, 2016). Furthermore, various enteric viral pathogens have progressively received interest as possible causal agents of runting-stunting syndrome (RSS) of chicken (Smyth *et al.*, 2010; Sajewicz-Krukowska *et al.*, 2016). RSS causes considerable loss of financial viability because of deprived feed adaptation, decreased body weight, size irregularity and reduction at slaughter, reduced

livability, hatchery condemnations and secondary diseases (Devaney *et al.*, 2016). Although growth retardation, reduced livability and hatchery condemnations are common in commercial poultry operations in Nigeria, they have not been linked to enteric viruses. This study therefore investigated commercial broilers and turkeys for enteric viruses associated with RSS in Ogun, Osun and Oyo States, Southwestern Nigeria, which is noted as the core of poultry industry in the country.

Chicken astrovirus (CAstV), ANV, ARV, ChPV and FAdV were detected in broiler chickens while CAstV was the only enteric virus detected in turkeys. Since vaccinations against these enteric viruses are not routinely practiced in commercial poultry in Nigeria, their detection shows natural exposure to them. In addition, detection of CAstV in turkeys indicates cross species transmission in the study area. This is supported by similar findings of CAstV in turkeys (Awe *et al*., 2015) and has been linked to diarrhoea as well as increased death in turkeys (Mettifogo *et al*., 2014). Furthermore, multiple alignments of obtained CAstV nucleotide sequences showed they were highly similar with 98-100% homology while phylogenetic analysis revealed they belonged to CAstV Bi clade.

Chicken astrovirus (CAstV) was detected in all the broilers with majority (62.5%) of them having high levels of viral RNA as well as sporadic detection of FAdV and ANV in day old chicks, and ANV, ChPV and ARV in the older birds. While CAstV was the only identified enteric virus in turkeys and this was detected in majority (83.5%) of the turkeys tested. These findings show that enteric viruses may infect all age groups of poultry and several studies worldwide have detected these viral enteric microbes in malabsorption diseases e.g. RSS in chickens (Smyth *et al.*, 2010; Zsak *et al*., 2013). However, the findings of the current study reiterate earlier propositions of CAstV being a major aetiology of RSS in poultry (Smyth, 2017; Kang *et al.*, 2018), since, not only were all the 164 broilers and 81 out of 97 turkey samples positive for only CAstV but the detected CAstV strain in these samples were virtually identical. This is unusual, given that usually there are in circulation several CAstV strains often with low shared genetic identity (Smyth *et al.*, 2012) and strongly implies that this particular strain is the cause of the hatchery condemnations and runting. The presence of similar CAstV strain in stunted older birds may indicate possibility of this virus to actually persist within flocks and could

perpetuate stunting particularly if it was contracted early. Additionally, this virus is less likely to have been contracted as later infections since chickens quickly develop resistance to the effects of CAstV and no strains have so far been identified that are primarily known to cause health issues or problems in older birds (Smyth *et al.*, 2012).

Recent studies have shown extensive incidences of CAstV infections in broilers particularly in connection with hatchery disease and infections of young poultry (Smyth *et al.*, 2013; Sajewicz-Krukowska *et al.*, 2016; Kang *et al*., 2018). Additionally, CAstV has been reported to be spread by feed and/or water contamination (i.e. horizontally) or vertically via naive in-lay parent birds (Nunez *et al.*, 2016). In the current study, only CAstV was identified in all the runted day-old broiler chicks, stunted 14-week-old broilers as well as nearly all of the runted day old turkey poults. Thus, the detection of CAstV in day- old broilers and turkeys shows vertical transmission of this virus and that these birds hatched shedding the virus and at high levels in many samples. Similarly, the detected CAstV in older birds may signify not totally cleared infection leading to continual infection. The high level of resistance of CAstV to regularly used disinfectants (Todd *et al.*, 2009) could have invariably contributed to this persistence in the older birds via likely constant contamination of the environment. Therefore, the detection of the virus in both day old chicks and poults, and older chickens indicates vertical transmission of CAstV in broilers and turkeys, and we hypothesize that the reduced health status of the older birds is due to early infection by this particular strain which caused stunting and has allowed it to persist in these birds at moderately maintained levels in South West Nigeria.

Avian nephritis virus, just like CAstV, is involved in regression of growth that may include irregular development as well as RSS, with reports of concurrent detection of both viruses in birds with the condition (Smyth *et al.*, 2010). We detected both viruses in day old and older broilers. However, there was a much less frequent detection of ANV than CAstV particularly in day-old chicks in the present study. This finding is in disparity with a rather larger detection of ANV-infected samples than CAstV-infected studies in Europe (Mettifogo *et al.*, 2014). Furthermore, ANV is known as a major recognized cause of baby-chick-nephropathy and thus anticipated to be found in ill hatchlings, although interestingly, CAstV rather than ANV was detected in all poorly or runted just hatched broilers as well as virtually every one of the poults in the present study. Thus, this finding

implicates the virus as a cause of the hatchery disease similar to the detection of CAstV in another hatchery condition- White Chick Syndrome (Smyth *et al.*, 2013).

Fast-growing broiler chickens are mainly predisposed to ChPV infections and the virus has been implicated as a cause of RSS and enteritis in chickens. Moreover, this virus is able to cause regressed growth, disorder in bone as well as abnormal plumes in broilers in natural or experimental infections mainly through early infections occurrence (Domanska-Blicharz *et al*., 2012; Zsak *et al*., 2013). Infections mainly occur around first weeks of hatch, but, in the present study, there was no ChPV detected in day-old chicks and poults. On the contrary, all of the six samples from the older (14 weeks) broiler chickens were positive. This may be due to the fact that birds infected void enormous quantities of the virus in the faeces that contributes to rapid and efficient bird-to-bird (horizontal) spread of disease (Zsak *et al*., 2013).

Avian reovirus, ANV, CAstV and FAdV are essential microbes of poultry causing growth underperformance as well as silent fatalities (Smyth *et al*., 2013). These viral pathogens are reported worldwide from bird flocks with growth failure, tenosynovitis, feathering abnormalities as well as enteritis (Zsak *et al*., 2013). The detection of these enteric viral pathogens in day old chicks in this study corroborates the fact that broiler birds are most susceptible to viral infections during the early post hatching period. This shows possible vertical transmission of these viral pathogens in the study area. Nevertheless, findings of CAstV, ChPV and ANV in adult birds show the possibility of infections persistence or horizontal spread.

Furthermore, ARV, ANV, ChPV and FAdV were only detected in sporadic samples. In this study, they were not prevalent whereas CAstV was in all the samples from day old and older broiler chickens and at elevated levels in nearly all the sampled chicks. In addition, the only detected enteric virus was CAstV in the sampled turkeys. With CAstV as the only detected virus that is present in every sample from day-old chick, older birds as well as nearly all samples from turkeys strongly suggests that it is the major cause of RSS in broilers in south west Nigeria. The consequences of enteric viral infection can impede the absorption competence of the intestinal tract during the first weeks of life of these birds, which increase depressed output throughout production phase. As such, the economic significance of these viruses especially CAstV in poultry primarily linked with

poor growth performance, treatment costs, reduced productivity and increased mortality cannot be overemphasized. Therefore, there is a need for focused bio-security and development of appropriate vaccination in broiler breeder flocks.

CHAPTER FIVE
SUMMARY AND CONCLUSIONS

5.1 Summary

High detection rate of chicken astrovirus and low occurrence of avian nephritis virus, avian reovirus, chicken parvovirus and fowl adenovirus in this study strongly suggest group Bi chicken astrovirus as the major causative agent of runting-stunting syndrome and hatchery condemnations in commercial broilers and turkeys in Southwestern Nigeria, although co-infections of chicken astrovirus and the other viruses cannot be ruled out

5.2 Conclusions

Findings from this study have established for the first time the involvement of avian reovirus (ARV), chicken astrovirus (CAstV), chicken parvovirus (ChPV), avian nephritis virus and fowl adenovirus (FAdV) as viral aetiology of runting-stunting syndrome in commercial broilers in Nigeria. Serodetection of CAstV 11672 in broilers and turkeys even at day-old has shown group B CAstV circulation in them. Isolation of enteric ARV and FAdV from broilers has been established in the study area. High detection rate of group Bi CAstV and low occurrence of ANV, ARV, ChPV and FAdV suggest that CAstV is strongly associated with runting-stunting syndrome and hatchery-condemnations in commercial broilers and turkeys in Southwestern Nigeria. Therefore, vaccination of layer and broiler breeders to eliminate vertical transmission of these viruses to prevent virus dissemination is advocated.

5.3 Contributions of this study to scientific knowledge

1. This study has established that runting-stunting syndrome in commercial broilers and turkeys in southwest Nigeria is caused by co-infection of enteric viruses (avian

reovirus, chicken astrovirus, chicken parvovirus, avian nephritis virus as well as fowl adenovirus) with chicken astrovirus being the predominant virus

2. Molecular studies established that the circulating chicken astrovirus strain in Nigeria belonged to the B1 genotype
3. Sequences of avian reovirus, chicken astrovirus, chicken parvovirus, avian nephritis virus as well as fowl adenovirus obtained from this study have been submitted to the GenBank database
4. Group B chicken astrovirus has been identified as the circulating serotype of chicken astrovirus in commercial broilers and turkeys in southwest Nigeria
5. This is the first cell culture isolation and ultra structural identification of fowl adenovirus in Nigerian poultry

REFERENCES

Adu, F.D. 2011.Cell culture in virology. *Viruses and their methods of identification*. Trafford publishing: USA. 42-51

Agri-Food and Biosciences Institute. 2012. Laboratory protocol for diagnostic tests. Protocol CC 114 versions1 and 2. 1-11

Aiki-Raji, C.O., Adebiyi, A.I., Agbajelola, V.I., Adetunji, S. A., Lameed, Q., Adesina, M., Adekanye, G., Omidokun, F., Fagbohun, O. and Oluwayelu, D.O. 2015. Surveillance for low pathogenic avian influenza viruses in live-bird markets in Oyo and Ogun States, Nigeria. *Asian Pacific Journal of Tropical Disease* 5.5: 369-373.

Awe, O.O., Kang, K.I., Ibrahim, M., Ali, A., Elaish, M., Saif, Y.M. and Lee, C.W. 2015. Age-related susceptibility of turkeys to enteric viruses. *Avian Diseases* 59:207-212

Balamurugan, V. and Kataria, J. M. 2004. The hydropericardium syndrome in poultry: a current scenario. *Veterinary Research Communication* 28:127–148

Barnes, H.J. and Guy, J.S. 2003. Poultry enteritis-mortality syndrome. Diseases of Poultry. Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L. R. and Swayne, DE, Eds. Ames, IA: State Press. 1171-1180

Baxendale, W. and Metbatsion T. 2004. Characterization and isolation of chicken astroviruses. *Avian Pathology* 33:364-370.

Bean, A.G.D., Baker, M.L., Stewart, C.R., Cowled, C., Deffrasnes, C., Wang, L-F. and Lowenthal, J.W. 2013. Studying immunity to zoonotic diseases in the natural host-keeping it real. *Nature Reviews: Immunology*13 (12):851–861

Benko, M., Harrach, B. and Russell, W.C. 2000. Family Adenoviridae. *Virus nomenclature*. Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B. Eds. International Committee on Taxonomy of Viruses, Seventh report. New York and San Diego: Academic press. 227–238.

Benko, M., Elo, P., Ursu, K., Ahne, W., LaPatra, S. E., Thomon, D. and Harrach. B. 2002. First molecular evidence for the existence of distinct fish and snake adenoviruses. *Journal of Virology* 76:10056–10059.

Berk, A.J., 2006. Adenoviridae: the viruses and their replication In “Fields Virology”, (D. M.K., P.M.H., P. Diane, E. Griffin, S. Robert, A. Lamb, M.A.M., B.R,. S.E.S., Eds.), Vol.2. 2 vols. Lippincott Williams & Wilkins

Bloom, M.E. and Kerr, J.R. 2006. Pathogenesis of parvovirus infections. *Parvovirus*. Kerr, J.R., Cotmore, S.F., Bloom, M.E., Linden, R.M. and Parish, C.R. Eds. UK: Hodden Arnolds. 323-341

Bosch, A., Guix, S., Krishna, N.K., Méndez, E., Monroe, S.S., Pantin-Jackwood, M. and Schultz-Cherry, S. 2012. Astroviridae. *Virus nomenclature*: International Committee on Taxonomy of Viruses, Ninth edition. Kings, A.M.Q., Lefkowitz, E., Adam, M.J. and Carsten, E.B. Eds. CA, USA: Elsevier. 953-959.

Breslin, J.J., Smyth, L.G., Barnes, H.J. and Guy, JS. 2000. Comparison of virus isolation, immunochemistry, and Reverse Transcriptase-Polymerase Chain Reaction procedures for detection of turkey- coronavirus. *Avian Diseases* 44:624-631

Cann, A.J. 2008. Replication of viruses. Section IV: Virus infection. *Desk encyclopedia of general virology*. Mahy, B.W.J. and Van-Regenmortel, M.H.V. Eds. USA: Academic Press. 315-322

Chorba, T.L. 2001. Disease surveillance. *Epidemiologic methods for the study of infectious diseases*. Thomas, J.C. and Weber, D.J. Eds. Oxford: Oxford U P. Chapter 7. 321-374

Cotmore,S.E. and Tattersall, P.C. 2006. Structure and organization of the viral genome. *Parvovirus*. Kerr, J.R., Cotmore, S.F., Bloom, M.E., Linden, R.M. and Parish, C.R. Eds. UK: Hodden Arnolds. 73-188

Daghir, N.J. 2008. Poultry production in hot climates. 2nd edition. Cambridge, MA; CAB International. 300-377.

Dai, Y., Xu, Q., Wu, X., Hu, G., Tang, Y., Li, J., Chen, Q. and Nie, J. 2010. Development of real-time and nested RT-PCR to detect astrovirus in one-year survey of astrovirus in Jiangmen City, China. *Archives of Virology* 155: 977–982.

Day, J.N., Spackman, E. and Pantin-Jackwood, M.J. 2007. A multiplex RT-PCR test for the differential identification of turkey astroviruses -1 and-2, avian nephritis virus, chicken astrovirus and avian rotavirus. *Avian Disease* 51:681-684

Day, J.M. and Zsak, L.2010. Determination and analysis of the full-length chicken parvovirus genome. *Virology* 399:59–64.

De Benedicts, P., Schultz- Cherry, A., Burnham, S. and Catoli, G. 2011. Astrovirus infections in humans and animals: molecular biology, genetic diversity, and interspecies transmissions. *Infection and Genetic Evolution* 11: 1529-1544.

De-la Torre, D.I, Nunez, L.F, Astolfi-Ferreira, C.S. and Ferreira, A.J.P. 2018. Enteric virus diversity examined by molecular methods in Brazilian poultry flocks. *Veterinary Science* 5:38

De Wit, J.J., Dam, G.B., de Laar, J.M., Biermann, Y., Verstegen, I., Edens, F. and Schrier, C.C. 2011. Detection and characterisation of a novel astrovirus in chicken and turkeys with enteric and locomotion disorders. *Avian Pathology* 40: 453-461

Decaesstecker, M., Charlier, G. and Muelemans, J. 1988. Epidemiological study of enteric viruses in broiler chickens: comparison of tissue culture and direct electron microscopy. *Avian pathology* 17:477-486.

Desselberger, U. 2000. Rotavirus: Basic facts. *Methods and procedures for rotaviruses*. Gray, J and Desselberger, U. Eds. Methods in Molecular Medicine 34:1-8

Devaney, R., Trudgett, J., Trudgett, A., Meharg, C. and Smyth, V.J. 2016. A metagenomic comparison of endemic viruses from broiler chickens with runting stunting syndrome and from normal birds. *Avian Pathology* 45: 616-629

Dey, B.K. 2003. Molecular epidemiologic studies on rotaviruses in calves, goats and poultry birds MSc Project. Dept. of Medicine, Agricultural University Mymensingh Bangladesh. Xix + 173

Dhama, K., Chauhan, R.S., Mahendran, M. and Malik, S.V.S. 2009. Rotavirus diarrhea in bovines and other domestic animals. *Veterinary Research Communication* 33:1-23.

Dhama, K., Saminathan, M., Karthik, K., Tiwari, R., Shabbir, M.Z., Kumar, N., Malik, Y.S. and Singh, R.J. 2015. Avian rotavirus enteritis-an updated review. *Veterinary Quarterly* 35.3: 142-158

Dimmock, N.J., Easton, A.J. and Leppard, K.N. 2016. Introduction to modern Virology. Hoboken, NJ: Blackwell publishing Ltd

Domanska-Blicharz, K.D., Bocian, L.I., Lisowska, A.B., Jacukowicz, A.J., Pikula, A.A. and Minta, Z.I. 2017. Cross-sectional survey of selected enteric viruses in Polish turkey flocks between 2008 and 2011. *BMC Veterinary Research* 13:108

Domanska-Blicharz, K., Jacukowicz, A., Lisowska, A. and Minta, Z. 2012. Genetic characterization of parvoviruses circulating in turkey and chicken flocks in Poland. *Archives of Virology* 157.12:2425-2430

Domanska-Blicharz, K., Tomczyk, G., Smietanka, K., Kozaczynski, W. and Minta, Z. 2011. Molecular characterization of fowl adenoviruses isolated from chickens with gizzard erosions. *Poultry Science* 90: 983-989.

Donato, C. and Vijaykrishna, Z. 2017. The broad host range and genetic diversity of mammalian and avian astroviruses. *Viruses* 9:102

Ercsey-Ravasz, M., Toroczkai, Z., Lakner, Z.and Baranyi, J. 2012. Complexity of the international agro-food trade network and its impact on food safety. *PLoS One* 7(5):e37810

FAOSTAT .2014.http://faostat3.fao.org/download/Q/QA/E. Retrieved May 15, 2016.

Fauci, A.S. and Morens, D.M. 2012. The perpetual challenge of infectious diseases. *New England Journal of Medicine* 366(5):454-461

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. 2005. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Springer : Vienna, Austria.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

Franco, M.A. and Greenberg H.B. 2009. Rotavirus. *Clinical virology*. Richman, D.D., Whitley, R.J. and Hayden, F.G. Eds. Washington DC: ASM press. 796-815

Gillespie, J.R. and Flanders, F.B. 2009. Modern livestock and poultry production. 8th edition. Clifton Park, New York: Delmar, Cengage Learning.1136.

Gough, R.E., Borland, E.D., Keymer, L.F. and Stuart, J.C. 1985. An outbreak of duck hepatitis type II in commercial ducks. *Avian Pathology* 14: 227–236.

Gough, R.E, Collins, M.S., Borland, E. and Keymer, L.F. 1984. Astrovirus-like particles associated with hepatitis in ducklings. *Veterinary Record* 114: 279

Huang, W.R., Wang, Y.C., Chi, P.I., Wang, C.Y., Lin, C.H. and Liu, H.J. 2011. Cell entry of avian reovirus follows a caveolin-1-mediated and dynamin-2-dependent endocytic pathway that requires activation of p38 mitogen-activated protein kinase (MAPK) and Src

signalling pathways as well as microtubules and small GTPase Rab5 protein. *Journal of Biological Chemistry* 286:30780–30794.

Ighodalo, E.T. and Akpavie, S.O. 2008. Runting and stunting syndrome in broiler chicks in Nigeria. Book of abstracts of 13th international congress on infectious diseases. *International Journal of infectious diseases* e135. Doi: 10.1016/j.ijid.2008.05.335.

Imada, T., Yamaguchi, S., Mase, M., Tsukamoto, K., Kubo, M. and Marooka, A. 2000. Avian Nephritis Virus as a new member of the family *Astroviridae* and construction of infectious ANV cDNA. *Journal of Virology* 74: 8487-8493

Jez, C., Beauamont, C. and Magdelaine, P. 2011. Poultry production in 2025: learning from future scenarios. *World's Poultry Science Journal* 67: 105-113.

Jiang, X., Crawford, S.E., Estes, M.K. and Prasad, B.V.V. 2008. Segmented double-stranded RNA viruses (rotavirus). *Molecular and structure biology*. Patton, J.T. Editor. Caister UK: Academic Press. 45-60

Jindal, N., Chander, Y., Patnayak, D.P., Mor, S.K., Ziegler, A.F. and Goyal, S.M. 2012. A multiplex RT-PCR for the detection of astrovirus, rotavirus, and reovirus in turkeys. *Avian Disease* 56:592-596.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* 451(7181):990–993

Jones, R.C. 2000. Avian reovirus infections. *Scientific and Technical Review of the Office International des Epizooties* 19.2: 614-625

Jones, R.C., Hughes, C.S. and Henry, R.R. 1979. Rotavirus infection in commercial layer-hens. *Veterinary Record* 104:22

Kang, K I., El-Gazzar, M., Sellers, H. S., Dorea, F., Williams, S. M., Kim, T., Collett, S. and Mundt, E. 2012. Investigation into the aetiology of runting and stunting syndrome in chickens. *Avian Pathology* 41:41–50.

Kang, K.I., Linemann, E., Icard, A.H., Durairaj, V., Mundt, E and Sellers, H.S.2018. Chicken astrovirus as an etiological agent of runting-stunting syndrome in broiler chickens. *Journal of General Virology* 99: 512-524

Kant, A., Blak, F. and Born, L. 2003. Classification of Dutch and German avian reoviruses by sequencing the σ C proteins. *Veterinary Research* 34: 203-212.

Karlsson, E.A., Small, C.T., Freiden, P., Feeroz, M.M., Matsen, F.A.T., San, S., Hasan, M.K., Wang, D., Jones-Engel, L. and Schultz-Cherry, S. 2015. Non-human Primates harbor diverse mammalian and avian astroviruses including those associated with human infections. *PLoS Pathogen* 11: e1005225

Kasamatsu, H. and Nakanishi, A. 1998. How do animal DNA viruses get to the nucleus? *Annual review of microbiology* 52:627-686

Kawaguchi, T., Nomura, K., Hirayama, T. and Kitagawa, T. 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH1. *Cancer Research* 4: 4460-4464.

Kemenesi, G., Dallos, B., Gorfol, T., Boldogh, S., Estok, P., Kurucz, K., Oldal, M., Nemeth, V., Madai, M., Banyai, K. and Jakab, F. 2014. Novel European lineages of bat astroviruses identified in Hungary. *Acta Virologica* 58:95–98

King, A.M.Q., Lefkowitz, E., Adams, M.J. and Carstens, E.B. 2012. Virus nomenclature. International committee on taxonomy of viruses, 9th edition. MA, USA: Academic press. 13-38

Kisary, J., Avalosse, B., Miller-Faures, A. and Rommelaere, J. 1985. The genomic structures of a novel chicken virus identify it as parvovirus. *Journal of General Virology* 66:259-263.

Koci, M.D. 2005. Immunity and resistance to astrovirus infection. *Viral Immunology* 18:11–16

Koci, M.D., Seal, B.S. and Schultz-Chery, S. 2000. Molecular characterisation of an Avian Astrovirus. *Journal of Virology* 74: 6173-6177

Koo, B., Lee, H., Jeon, E., Han, M., Min, K., Lee, S. and Mo, P. 2013. Molecular survey of Enteric Viruses in chicken farms in Korea with a history of enteritis. *Poultry Science* 92: 2876-2885

Kort, Y.H., Bourogaa, H., Gribaa, L., Scott-Algara, D. and Ghram, A. 2013. Molecular characterization of avian reovirus isolates in Tunisia. *Virology Journal* 10:12

Krishna, N. K. 2005. Identification of structural domains involved in astrovirus capsid biology. *Viral Immunology* 18:17–26

Krishnan, T. 2014. Novel human astroviruses: Challenges for developing countries. *Virus Disease* 25: 208–214.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

Kuss, S.K., Best, G.T., Etheredge, C.A., Pruijssers, A.J., Frierson, J.M., Hooper, L.V., Dermody, T.S. and Pfeiffer, J.K. 2011. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science* 334:249–252.

Liu, H., Lee, H., Shih, L., Li, Y. and Su, H.2004. Rapid characterisation of avian reovirus using phylogenetic analysis, reverse transcription-polymerase chain reaction and restriction enzyme fragment length polymorphism. *Avian Pathology* 33:171–180.

Long, K.E., Hastie, G.M., Ojkic, D. and Brash, M.L. 2017. Economic impacts of white chick syndrome in Ontario, Canada. *Avian Disease* 61: 402-408

MacLachlan, N.J. and Dubovi, E.J. 2011. Astroviridae. Epidemiology and Control of Viral Diseases. *Fenner's Veterinary Virology*. London UK: Academic Press, 4th edition. 142-454

Madeley, C.R. and Cosgrove, B.P. 1975. 28 nm particles in faeces in infantile gastroenteritis. *The Lancet* 6: 451-452

Martella, V., Banyai, K., Matthijnssens, J., Buonavoglia, C. and Ciarlet, M. 2010. Zoonotic Aspects of Rotaviruses. *Veterinary Microbiology* 140;246-255

Matsui, S.M. and Greenberg, H.B. 2001.Astroviruses. Knipe, D.M., Howley, P.M. eds. *Fields Virology* 4th edn. Baltimore, MD: Lippincott, Williams & Wilkins 1: 875-893.

McNeilly, F., Connor, T.J., Calvert, V.M., Smyth, J.A., Curran, W.L., Morley, A.J., Thompson, D., Singh, S., McFerran, J.B., Adair, B.M. and McNulty, S. 1994. Studies on a novel enterovirus like virus isolated from chickens. *Avian Pathology* 23:313-327

McNulty M.S. 2003. Rotavirus infection. *Diseases of poultry*. Saif, Y.M, Barnes, H.J, Glisson, J.R, Fadly, A.M, McDougald, L.R, Swayne, D.E, Eds. Ames (IA): Iowa state press.308-317.

McNulty M.S., Curran W.L. and McFerran J.B. 1980. Detection of astrovirus in turkey feces by electron microscopy. *Veterinary Record* 106: 561.

Mettifogo, E., Nu˜nez, L. F., Chac´on, J. I., Santader-Parra, S. I.H., Astolfi-Ferreira, C. S., Jerez, J. A., Jones, R. C. and Ferreira, A. J. P.2014. Emergence of enteric viruses in production chickens is a concern for avian health. *Scientific World Journal* 2014: 450423.

Meulemans, G., Boschmans, M., Berg, V. D. and Decaesstecker, M. 2001. Polymerase chain reaction combined with restriction enzyme analysis for detection and differentiation of fowl adenoviruses. *Avian Pathology* 30:655–660.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T.L., Agarwala, R. and Schäffer, A.A. 2008. Database Indexing for Production MegaBLAST Searches. *Bioinformatics* 24:1757-1764.

Murphy, F.A., Gibbs, E.P.J., Horzinek, M.C. and Studdert, M .J. 2005. *Veterinary Virology* 3rd edition. CA , USA: Academic press. 461-475

Muzyczka, N. and Berns, K.I. 2011. Parvoviruses and their replication. *Fields Virology*, 4th edition, volume two. Knipe, D.M. and Howley, P.M. Eds. USA: Lippincott, Williams and Wilkins. 2327-2360

Noah, N.2008. Disease Surveillance. In: Encyclopedia of Virology (3 rd edition) (Mahy, B.W.J. and Van Regenmortel, M.H.V. Eds.). Elsevier publishers, USA

Nuñez, L.F.N., Santander Parra, S.H., Carranza, C., Astolfi-Ferreira, C.S., Buim, M.R. and Piantino Ferreira, A.J. 2016. Detection and molecular characterization of chicken astrovirus associated with chicks that have an unusual condition known as "white chicks" in Brazil. *Poultry Science* 95: 1262–1270

Olsen, D.E. 1977. Isolation of a reovirus-like agent from broiler chicks with diarrhoea and stunting. Proceedings of the 26th Western Poultry Diseases Conference. Davis, California. 131-139

Oluwayelu, D.O. and Adebiyi, A.I. 2015. Possible mechanisms underlying bacterial-viral interactions in respiratory diseases: a review. *African Journal of Infectious Diseases* 9.2:43-46

Oluwayelu, D.O., Adebiyi, A.I., Olaniyan, I.T., Ezewele, P, and Aina, O. 2014. Occurrence of Newcastle Disease and Infectious Bursal Disease Virus Antibodies in Double-spurred Francolins in Nigeria. *Journal of Veterinary Medicine,* 2014, 106898, doi: 10.1155/2014/106898

Oluwayelu, D.O. and Todd, D. 2012. Chicken astrovirus infection: Mini review and preliminary serologic evidence of antigenically and genetically distinct chicken astroviruses in Nigerian indigenous chickens. *African Journal of Biomedical Research* 15.2: 71-76.

Oluwayelu, D.O., Smyth, V. and Todd, D. 2011. Detection of chicken astrovirus and avian nephritis virus in Nigerian indigenous chickens. *African Journal of Biotechnology* 11.17: 3949-3957.

Pantin-Jackwood, M.J., Day, J. M., Jackwood, M.W. and Spackman, E. 2008. Enteric viruses detected by molecular methods in commercial turkey and chicken flocks in the United States, 2005 and 2006. *Avian Disease* 52:235-244

Patton, J.T., Silvestri, L.S., Tortorici, M.A., Vasquez-Del Carpio, R. and Taraporewala, Z.F. 2006. Rotavirus genomic replication and morphogenesis: Role of the viroplasms. *Entry, assembly and morphogenesis of reovirus*. Roy P. Editor. Germany: Springer press. 169-187.

Pesavento, J.B., Crawford, S.E., Estes, M.K. and Prasad, B.V. 2006. Proteins structure and assembly of rotavirus. *Current Topics in Microbiology and Immunology* 309:189-219.

Radwan, M.M., El-Deeb, A.H., Mousa, M.R., El-Sanousi, A.A. and Shalaby, M.A. 2018. First report of fowl adenovirus 8a from commercial broiler chickens in Egypt: molecular characterization and pathogenicity. *Poultry Science* 10:1-8

Ramphal, R., Samal, P.M., Shands, W., Fischlschweiger, W. and Small, P.A. 1980. Adherence of Pseudomona aeruginosa to tracheal cells injured by influenza infection or by endotracheal intubation. *Infection and Immunology* 27.2:614-619.

Reynolds, D.L. and Schultz-Cherry, S.L. 2008.Astrovirus infections. *Diseases of Poultry*. Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K. and Swayne, D.E. Eds. Ames, IA USA: Blackwell. 351–355

Rist, C.L., Arriola, C.S. and Rubin, C. 2014. Prioritizing zoonoses: a proposed One Health tool for collaborative decision-making. *PLoS One* 9(10): e109986

Rosenberger, J.2012. Update on the runting-stunting syndrome. Ceva Eggs Program Online. 2012. Retrieved on December 25 2017. http://fs-1.5mpublishing.com/images/ceva/EPO_No3-May2012.pdf

Roussan, D.A., Shaheen, I.A., Khawaldeh, G.Y., Totanji, W.S. and Al-Rifai, R.H. 2012. Simultaneous detection of astrovirus, rotavirus, reovirus and adenovirus type I in broiler chicken flocks. *Polish Journal of Veterinary Sciences* 15.2:337-344

Roy, P., Koteeswaran, M. and Manickam, R. 1999. Efficacy of an inactivated oil emulsion vaccine against hydropericardium syndrome in broilers. *Veterinary Record* 145:458–459.

Saif, Y.M.2008. Viral enteric infections. *Diseases of Poultry.* Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K. and Swayne, D.E. Eds. Ames, Iowa; USA: Blackwell.329–330.

Saif, Y.M.2013. Viral enteric infections. *Diseases of Poultry*, 13 edition. Swayne, D.E., Ed. Florida USA: John Wiley and Sons Inc. Chapter 12.375-389

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.

Sajewicz-Krukowska, J. and Domanska-Blicharz, K. 2016. Nearly full-length genome sequence of a novel astrovirus isolated from chickens with 'white chicks' condition. *Archives of Virology* 161: 2581–2587

Salsman, J., Top, D., Boutilier, J. and Duncan, R. 2005. Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability. *Journal of Virology* 79:8090–8100

Schat, K.A. and Purchase, H.G. 1998. Cell-culture methods. *A laboratory manual for the detection of avian microbes*, 4th edition. Florida, USA: Rose printing, Tallahasse. 223-234

Schneider, W.L. and Roossinck, M.J. 2001. Genetic diversity in RNA virus quasi-species is controlled by host-virus interactions. *Journal of Virology* 75: 6566–6571.

Schnitzer, T.J. 1985. Protein coding assignment of the S genes of the avian reovirus S1133**.** *Virology* 141: 167-170.

Schultz-Cherry, S., King, D.J. and Koci, M.D. 2001. Inactivation of an astrovirus associated with poult enteritis mortality syndrome. *Avian Diseases* 45: 76–82.

Sellers, H., Linneman, E., Icard, A.H. and Mundt, E. 2010. A purified recombinant baculovirus expressed capsid proteins of a novel astrovirus provides partial protection to Runting-Stunting Syndrome in chicken. *Vaccine* 28:1253-1263

Shih, W.L., Hsu, W.H., Liao, M.H., Lee, L.H. and Liu, H.J. 2004. Avian reovirus σ C protein induces apoptosis in cultured cells. *Virology* 32:65–77.

Smyth, V.J., Jewhurst, H.L., Adair, B. and Todd, D.2009.Detection of Chicken Astrovirus by Reverse Transcriptase-Polymerase Chain Reaction. *Avian Pathology* 38:293-299

Smyth, V.J. 2017. A review of the strain diversity and pathogenesis of chicken astrovirus. *Viruses* 9: 29

Smyth, V.J., Jewhurst, H.L., Wilkinson, D.S., Adair, B.M., Gordon, A.W. and Todd, D. 2010. Development and evaluation of real time TaqMan® RT-PCR assays for the detection of Chicken Astrovirus and Avian Nephritis Virus in chickens. *Avian Pathology* 39: 467-474.

Smyth, V.J., Kaukonen, E., Trudgett, J., Wylie, M., Jewhurst, H., Conway, B., Welsh, M.D. and Todd, D. 2013. Chicken astrovirus detected in hatchability problems associated with "White Chicks". *Veterinary Record* 173: 403–404

Smyth, V.J., Todd, D., Trudgett, J., Lee, A. and Welsh, M.D. 2012. Capsid protein sequence diversity of chicken astrovirus. *Avian Pathology* 39: 151–159.

Soares, R.M., Durigon, E.L., Bersano, J.G. and Richtzenhain, L.J. 1999. Detection of porcine parvovirus DNA by the polymerase chain reaction assay using primers to the highly conserved nonstructural protein gene, NS1. *Journal of Virological Methods* 78: 191-198

Spackman, E., Day, J.M. and Pantin-Jackwood M.J. 2010. Astrovirus, rotavirus and reovirus concomitant infections cause decreased weight-gain in broad breasted white Poults. *Avian Disease* 54:16-21

Strother, K.O., and. Zsak, L. 2009. Development of Enzyme-linked Immunosorbent Assay to detect chicken parvovirus specific antibody. *Avian Disease* 53: 585 -591.

Takase, K., Shinohara, K., Tsuneyoshi, M., Yamamoto, M. and Yamada, S. 1989. Isolation and characterisation of cytopathic avian enterovirus from broiler chickens. *Avian Pathology*18: 631-642

Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by the neighbour-joining technique. *Proceedings of National Academy of Sciences (USA)* 101:11030-m11035.

Tarasiuk, K. Wozniakowski, G. and Samorec-Salamonowiz, E. 2012. Occurrence of Chicken Parvoviral infections in Poland. *The open Virology Journal* 6:7-11.

Tattersall, P., 2006. The evolution of parvovirus classification. *Parvovirus*. Kerr, J.R., Cotmore, S.F., Bloom, M.E., Linden, R.M. and Parish, C.R. Eds. UK: Hodden Arnolds. 5-14.

Tijssen, P., Agbandje-McKenna, M., Almendral, J.M., Bergoin, T.W., Flegel, M., Hedman, K., Kleinschmidt, J., Li, Y., Pintel, D.J., and Tattersall, P. 2012. *Parvoviridae*. International Committee on Taxonomy of Viruses, 9th edition. King, A.M.Q., Adams, MJ, Carstens, E.B. and Lefkowitz, E.J. Eds. UK: Academy press. 405-425

Todd, D., Trudgett, J., Smyth, V.J., Donelly, B., McBride, N. and Welsh, M.D. 2011. Capsid protein sequence diversity of avian nephritis virus. *Avian pathology* 40.3: 249-259

Todd, D., Wilkinson, D.S., Jewhurst, H.L., Wylie, M., Gordon, A.W. and Adair, B.M. 2009. A Seroprevalence investigation of Chicken Astrovirus infection. *Avian Pathology* 38: 301-309

Taylor, L.H., Latham, S.M. and Woolhouse, M.E.J. 2001. Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 356 (1411):983–989

Trampel, D.W., Kinden, D.A., Solorzano, R.F. and Stogsdill, P.L. 1983. Parvovirus-like enteropathies in turkeys, Missouri. *Avian Diseases* 27:49-54

Van Regenmortel, M.H.V., Fauquet, C.M. and Bishop, D.H.L. 2000. Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses. New York: Academic Press.

Veen, T.C., De Bruijn, N.D., Dijkman, R. and De Wit, J.J. 2017. Prevalence of histopathological intestinal lesions and enteric pathogens in Dutch commercial broilers with time. *Avian Pathology* 46:95-105.

Willcocks, M. M., Brown, T. D.K., Madeley, C.R. and Carter, M.J. 1994. The complete sequence of a human astrovirus. *Journal of General Virology* 75.7:1785-1788.

Woo, P.C., Lau, S.K., Teng, J.L., Tsang, A.K., Joseph, S., Xie, J., Jose, S., Fan, R.Y., Wernery, U. and Yuen, K.Y. 2015. A novel astrovirus from dromedaries in the Middle East. *Journal of General Virology* 96: 2697–2707

Yamaguchi, S., Imada, T. and Kawamura, H. 1979. Characterization of a picornavirus isolated from broiler chicks. *Avian Disease* 23: 571–581.

Yason, C.V. and Schat K.A. 1986. Experimental infections of SPF-chickens with avian rotavirus. *Avian Disease* 30:551-556.

Yason, C.V., Summers, B.A. and Schat K.A. 1987. Pathogenesis of rotaviral infections in different age group of chickens and turkeys: pathology. *American Journal of Veterinary Research* 48:927–938.

Zavala, G. and T. Barbosa. 2006. Runting and stunting in broiler chickens. Apinco-Facta, May 2006. Retrieved October 17 2017. http://www.poultryhealth.com/fora/inthelth/zavala_apinco_06. pdf.

Zhang, Y., Liu, M., Shuidong, O., Hu, Q.L., Guo, D.C., Chen, H.Y. and Han, Z. 2006. Detection and identification of avian, duck, and goose reoviruses by RT-PCR: goose and duck reoviruses are part of the same genogroup in the genus Orthoreovirus. *Archives of Virology* 15:1525–1538.

Zhao, W, Zhu, AL, Yuan, CL, Yu, Y, Zhu, CX, Lan, DL, Yang, ZB, Cui, L and Hua, XG. 2011. Detection of astrovirus infection in pigeons (*Columbia livia*) during an outbreak of diarrhoea. *Avian Pathology* 40: 361–365.

Zsak, L., Cha, R.M. and Day, J.M. 2013.Chicken parvovirus induced runting-stunting syndrome in broilers. *Avian Diseases* 57: 123 – 127.

Zsak, L, Strother, K.O. and Day, J.M. 2009. Development of a Polymerase Chain Reaction procedure for detection of chicken and turkey parvoviruses. *Avian Diseases* 53:83-88.

Appendix

UNIVERSITY OF IBADAN
ANIMAL CARE AND USE RESEARCH ETHICS COMMITTEE
(UI-ACUREC)

E.mail: animaluseresearch@gmail.com/ animaluseresearch@yahoo.com

08176917269

Ref: ..... Date: 19-3-19

Assigned Number: UI-ACUREC/18/0116

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Investigation of Enteric Viruses Associated with Runting and Stunting Problems in Commercial Broilers and Turkeys in Southwest Nigeria

Name and Address of Principal Investigator: Adebowale Idris Adebiyi
Department of Veterinary Microbiology
Faculty of Veterinary Medicine
University of Ibadan, Ibadan

Date of receipt of valid application: 29/10/2018

Date of meeting when final determination on ethical approval was made: 26/2/2019

This is to inform you that the research described in the submitted protocol, have been received and *given full approval by the* UI-ACUREC.

This approval dates from **26/2/2019 to 25/2/2020.** If there is delay in starting the research, please inform UI-ACUREC so that the dates of approval can be adjusted accordingly.

Note that no activity related to this research may be conducted outside of these dates. It is expected that you submit your annual report as well as an annual request for the project renewal to the UI-ACUREC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenet of the code including ensuring that all adverse events are reported promptly to the UI-ACUREC. No changes are permitted in the research without prior approval by the UI-ACUREC except in circumstances outlined in the code.

You are to note that UI-ACUREC reserves the right to monitor and conduct compliance visit to your research site without previous notification.

Prof. S. I. B. Cadmus
Chairman, UI-ACUREC