# PREVALENCE, IDENTIFICATION AND GENETIC DIVERSITY OF *EIMERIA* SPECIES IN COMMERCIAL POULTRY FARMS IN ZARIA AND ENVIRONS, KADUNA STATE, NIGERIA

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# PREVALENCE, IDENTIFICATION AND GENETIC DIVERSITY OF *EIMERIA* SPECIES IN COMMERCIAL POULTRY FARMS IN ZARIA AND ENVIRONS, KADUNA STATE, NIGERIA

**BY**

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**DEPARTMENT OF VETERINARY PARASITOLOGY AND ENTOMOLOGY, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

**NOVEMBER, 2014**

## DECLARATION

I declare that the work in this Dissertation entitled "**Prevalence, Identification and Genetic Diversity of *Eimeria* species in Commercial Poultry Farms in Zaria and Environs, Kaduna State, Nigeria**" has been carried out by me in the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Isa Danladi JATAU _____ _____

Signature Date

## CERTIFICATION

This dissertation entitled**, "PREVALENCE, IDENTIFICATION AND GENETIC DIVERSITY OF *EIMERIA* SPECIES IN COMMERCIAL POULTRY FARMS IN ZARIA AND ENVIRONS KADUNA STATE, NIGERIA"** by Isa Danladi JATAU, meet the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation**.**

| | | |
|---|---|---|
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## ABSTRACT

The use of molecular technique is the most accurate and reliable way of identification and characterization of natural infection by *Eimeria* species in chickens. In Nigeria, information on the characterization of chicken *Eimeria* are mainly based on the subjective and unreliable parasitological methods. This study determined the prevalence and identify the *Eimeria* species occurring in commercial poultry farms in Zaria and environs using oocysts morphometric and molecular techniques. It also determined the extent of genetic diversity in the internal transcribed spacer-1 and -2 (ITS-1 and -2) of the isolated *Eimeria* species, apical membrane antigene-1 coding gene of *Eimeria tenella* isolates (EtAMA-1) and whole genome of an *E. tenella* isolate. Litter samples were collected from 123 farms to survey for *Eimeria* oocysts. Out of the 37 (30.1%) positive samples, 14 were selected for morphometric and molecular identification of *Eimeria* species and the genetic diversity determination. The morphometric identification was done by the determination of shape index (length/width) of randomly selected oocysts. A nested PCR was used for the molecular identification of common chicken *Eimeria* (ChE) while the new operational taxonomic units (OTUs) of ChE were surveyed using ITS-2 based PCR. Amplification by PCR, cloning and sequencing of ITS -1 and -2 loci was also done for molecular identification of ChE including the OTUs. Genetic diversity among the ITS -1 and -2 sequences of the isolated *Eimeria* species were determined using phylogenetic and principal co-ordinate analyses in comparison with sequences of isolates from different parts of the world. Species-specific characterisation focused on the EtAMA-1 coding locus was done by the amplification, cloning and sequencing of the targeted locus for determination of genetic polymorphism among the local clones in comparison with isolates from different parts of the world. Whole genome next generation sequencing of an *Eimeria tenella* isolate was carried out using Illumina Hiseq2000 technology. The sequence data generated were added to those generated

elsewhere from Wisconsin (US) and Nippon (Japan) strains of *E. tenella* and then compared with the 2007 *E. tenella* Houghton strain reference genome assembly. There was no statistically significant association between the prevalence of the *Eimeria* infection and specific locations of the flocks ($\chi^2$ = 7.587, P > 0.05) and flock type ($\chi^2$ = 1.856, P > 0.05). The prevalence of the infection was statistically associated with flock age ($\chi^2$ = 6.406, P < 0.05); flock size ($\chi^2$ = 9.162, P < 0.05) and management system ($\chi^2$ = 4.579, P < 0.05). The morphometric identification method revealed all the seven ChE, while the presence of *E. tenella, E. maxima*, *E. necatrix*, *E. acervulina*, *E. mitis* and OTUs (OTU-X, OTU-Y and OTU-Z) were confirmed for the first time using molecular diagnostic tools in the study area. *Eimeria brunetti* and *E. praecox* were not detected using the molecular techniques. There was widespread occurrence of inter- and intra-specific genetic diversity among the ITS sequences of the isolated *Eimeria* species and in comparison with isolates from different parts of the world, apparently independent of geographic origin. Genetic diversity was more limited among EtAMA-1 alleles of wild *E. tenella* isolates from within and outside the country, with minimal diversity compared to archived laboratory strains. The whole genome sequence of the wild Nigerian strain of *E. tenella* was more genetically diverse from the reference H strain than either of the other laboratory strains (Wisconsin and Nippon). The implications of the observed genetic diversity among the studied *Eimeria* species on the prospect of future development of recombinant anticoccidia vaccines were discussed. It is recommended that poultry farmers in the study area should imbibe the practice of proper biosecurity, good sanitation and litter management as well as avoidance of other factors that will favour sporulation and dissemination of *Eimeria* oocysts in order to prevent outbreak of coccidiosis. Further detailed study on the identified OTUs is recommended to established their specific status and importance as pathogens in chickens.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| AMA-1 | Apical Membrane Antigene-1 |
| AP-PCR | Arbitrarily Primed-Polymerase Chain Reaction |
| BLAST | Basic Local Alignment Search Tool |
| Bp | Base pairs |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CE | Capillary Electrophoresis |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylene Diamine Tetra Acetate |
| EST | Expressed Sequence Tags |
| EtAMA-1 | Eimeria tenella Apical Membrane Antigene-1 |
| gDNA | Genomic Deoxyribonucleic Acid |
| GPI | Glycosylphosphatidylinositol |
| IFN-γ | Gamma Interferon |
| IL-6 | Interleukin-6 |
| IMP-1 | Immune Mapped Protein-1 |
| ITS | internal transcribed spacer |
| Kbp | Kilo base pairs |
| LINE | long interspersed nuclear elements |

| | |
|---|---|
| LTR | Long Terminal Repeat |
| Mbp | Mega base pairs |
| MEE | Multilocus Enzyme Electrophoresis |
| MEGA | Molecular Engineering Genetic Analysis |
| MHC-II | Major Histocompatibility Complex type II |
| MICs | Microneme proteins |
| NCBI | National Center for Biotechnology Information |
| OTU | Operational Taxonomic Unit |
| PABA | Para Amino Benzoic Acid |
| PBS | Phosphate Buffered Saline |
| PCoA | Principal Co-ordinate Analysis |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulsed Field Gel Electrophoresis |
| PV | Parasitophorous Vacuole |
| RAPD | Random Amplified Polymorphic DNA |
| rDNA | Ribosomal Deoxyribonucleic Acid |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic acid |
| RONs | Rhoptry neck proteins |
| ROPs | Rhoptry proteins |

| | |
|---|---|
| rRNA | Ribosomal Ribonucleic Acid |
| RVC | Royal Veterinary College |
| SAGs | Surface Antigens |
| SCARs | Sequence-Characterized Amplified Regions. |
| SNP | Single Nucleotide Polymorphism |
| SSCP | Single Strand Conformation Polymorphism |
| TAE | Tris Acetate EDTA |
| TCR | T-cell receptor |
| TRAP | Thrombospondin-Related Anonymous Protein |
| TSP I | Thrombospondin type I |

# CHAPTER ONE

# GENERAL INTRODUCTION

## 1.1 Introduction

Avian coccidiosis is an enteric parasitic disease caused by multiple species of protozoan parasite of genus *Eimeria* and is one of the commonest and economically most important diseases of poultry world-wide; causing production losses, high morbidity (due to acute, bloody enteritis) and mortality rates (Shirley *et al*., 2005). Seven species of *Eimeria* are widely recognized as the causative agents of coccidiosis in chickens, of which *E. tenella, E. necatrix, E. maxima,* and *E. brunetti* are highly pathogenic, *E. acervulina* and *E. mitis* are less pathogenic, whilst *E. praecox* is regarded as the least pathogenic (McDougald, 2003; Shirley *et al*., 2005; Conway and McKenzie, 2007; Taylor *et al*., 2007). In Nigeria, Majaro (1981) reported and described the seven *Eimeria* species in exotic commercial broiler chickens. Six *Eimeria* species were also identified by Majaro (1993) in indigenous domestic fowls and these included: *E. acervulina, E. tenella, E. brunetti, E. maxima, E. necatrix* and *E. mitis.* Recent molecular studies on the epidemiology of coccidiosis and genetic structures of *Eimeria* populations on commercial chicken establishments have identified genetic variants of *Eimeria* (i.e new operational taxonomic units OTU-X, OTU-Y and OTU-Z) that are genetically distinct from species of *Eimeria* identified previously in chickens (Morris *et al*., 2007; Cantacessi *et al*., 2008). According to Cantacessi *et al*. (2008) and Fornace *et al*. (2013) OTU-X represents a population variant of *E. maxima* while OTU-Y and OTU-Z represent cryptic species of *Eimeria*.

Transmission of *Eimeria* is mediated via an environmentally resistant oocyst and infection occurs when a susceptible chicken ingests the sporulated oocyst. Infective oocysts could be spread by contaminated equipment, dust, people, rodents, wild birds as

well as insects (Champman, 1997; Majaro, 2001). Distribution and prevalence of avian coccidiosis is influenced by several factors: high poultry density cramped in a small space, high air temperature, high relative humidity, mixing birds of different age categories at the same place, birds genotype, feed change, quality of feed, and other factors that compromise resistance to the disease and general health status of the birds (McDougald, 2003). In Nigeria, incidence of coccidiosis outbreaks is generally more common during the hot wet season than during cooler dry season. However, in intensively managed birds outbreaks may occur at any season of the year (Adene and Oluleye, 2004; Etuk *et al.*, 2004; Abdu, 2007).

Diagnosis of coccidiosis is traditionally based on gross lesions in the intestine, as well as microscopically using the contents of the intestine as a sample (McDougald, 2003). Intensity of infection can also be estimated especially if there is a doubt whether coccidia are the only cause of the disease. However, due to the limitation of the traditional methods of diagnosis of coccidiosis of being subjective and unreliable in the diagnosis of mixed natural infections (Long and Joyner, 1984; Woods *et al*., 2000a; Williams, 2001); several molecular techniques have been developed for more accurate diagnosis of coccidiosis (Procunier *et al*., 1993; Fernandez *et al*., 2003b; Lew *et al*., 2003; Su *et al*., 2003; Gasser, 2006).

Coccidiosis can be treated with anticoccidials which act either as coccidiostats, that inhibit growth and development of the intracellular parasite form or coccidiocides which kill the parasites during the developmental stages (Long and Jeffers, 1986). Since anticoccidial resistance develops rapidly and is now widespread, vaccination is the most

appropriate method for controlling coccidiosis since immunized birds develops solid immunity against homologous infection (Augustine *et al.,* 2001).

Vaccination against coccidiosis is the most effective way to achieve immunoprophylaxis, although production limitations, including a requirement for *in vivo* passage, limit production capacity and elevate cost. Most current vaccines are based on varied formulations of live parasites. In this way the immune system is activated so that subsequent natural infection causes a secondary immune reaction which is faster and better in comparison to the primary immune reaction (Dimitrijevi and Ilić, 2003). However, proper selection and production of effective and cheaper subunit or recombinant vaccines against coccidiosis will require better understanding of the population diversities of *Eimeria* species within a locality or geographical region.

The present study describes and characterise at molecular level the common *Eimeria* species occurring in commercial poultry farms in Zaria metropolitan areas and it's environ. The study also characterized a novel vaccine candidate genes the apical membrane antigene-1 (AMA-1) of *Eimeria tenella* isolates from the study area.

### 1.2 Statement of Research Problem

Records have shown that among the animal production modules, the poultry sector is now the fastest growing. What started as backyard "village poultry industries" during the mid-20th century have evolved into skilful and organized agribusinesses in many developing countries (FAO, 1998; Adene and Oluleye, 2004). Thus, poultry production has greatly expanded in many developing countries in the recent past and expansion is predicted to continue for at least 30 years in Africa and Asia (Blake and Tomley, 2014).

Unfortunately, the poultry industry has been adversely affected by a variety of constraints (McDougald, 2003; Adene and Oluleye, 2004). Of these constraints, diseases play a leading role in hampering the development of poultry production (Rushton *et al.*, 1999; Haug *et al*., 2008a; McDougald and Fitz-Coy, 2008). A major and recurring disease in poultry is coccidiosis (Shirley *et al*., 2005; Chapman *et al*., 2013). Coccidiosis is one of the most important poultry diseases that cause considerable economic losses in the poultry industry, and appears to be the most frequently reported disease of chickens worldwide (Biggs, 1982). The economic losses produced by coccidiosis worldwide have been reported to be about 2.4 billion US dollars (Shirley *et al*., 2005). The annual cost of medication against coccidiosis alone exceeds 300 million US dollars worldwide (McDougald and Fitz-Coy, 2008).

Epidemiological studies in Nigeria, have established the economic importance of coccidiosis as a major parasitic disease of poultry (Majaro, 1980; 1983; 2001; Adene and Oluleye, 2004; Abdu, 2007). The importance of coccidiosis is based on the economic implications of its outbreak in poultry farms (Majaro, 1980; Barksh, 1989) and to a greater extents due to the subclinical form of the disease (Williams, 1999; Haug *et al*., 2008a). Losses due to subclinical coccidiosis manifests as poor commercial performance of the bird because of poor nutrient utilization (Williams, 1999; Haug *et al*., 2008a). Studies conducted on outbreaks of coccidiosis in Zaria, Nigeria, suggest the presence of *E. tenella* and *E. necatrix*, two of the most pathogenic *Eimeria* species in chickens (Bishu, 1982). Outbreaks of coccidiosis in commercial poultry with up to 50% mortality were reported in Zaria, Nigeria (Abdu *et al*., 2008; Musa *et al*., 2010). Coccidiosis occur throughout the year in Northern Nigeria, with higher prevalence rates from May to September (Etuk *et al*., 2004; Abdu, 2007). Despite the significant economic impact of

coccidiosis, there is paucity of information using reliable molecular tools on the occurrence, distribution, morphological and genetic characteristics of *Eimeria* species in commercial poultry farms in Nigeria. Such information is vital for proper diagnosis and control of coccidiosis.

Owing to the widespread occurrence of drug resistant parasites and difficulties associated with the production and use of live vaccines, new approaches of controlling coccidiosis are urgently needed (Innes and Vermeulen, 2006;Williams, 2006; Morris *et al*., 2007). Recent efforts to clone genes from *Eimeria* spp. for potential recombinant vaccines are directed toward developing an alternative strategy for control of the parasite (Dalloul and Lillehoj, 2006; Blake and Tomley, 2014).

The genomes of protozoan parasites encode many thousands of genes and hence represent a large and complex pool of potential antigens (Gardner *et al*., 2002; Abrahamsen *et al*., 2004; Xu *et al*., 2004; Berriman *et al*., 2005; El-Sayed *et al*., 2005; Ivens *et al*., 2005; Pain *et al*., 2005; ). Molecules encoded within the *Eimeria* genome capable of stimulating a protective immune response during natural infection form an attractive target for the development of recombinant subunit vaccination strategies, and indeed, many vaccine candidate genes have been investigated (Subramanian *et al*., 2008; Blake *et al*., 2011a; Jiang *et al*., 2012; Oakes *et al*., 2013; Yin *et al*., 2013).

Although several recombinant vaccine candidate proteins have been proposed, effective recombinant subunit vaccines have not yet been formulated (Vermeulen *et al* 2001; Shirley *et al.,* 2007; Han *et al*., 2010) due to the technical difficulty of differentiating immunogenicity from real immune protection (Blake *et al*., 2011a). To develop effective

recombinant vaccines, it is crucial to characterize various components of the parasite and to understand the nature of the host–parasite interaction (Jiang *et al*., 2012).

Recently, studies on molecular and immunological interaction between the host and *Eimeria* have identified a panel of immunoprotective antigens such as apical membrane antigen 1 (AMA-1), immune mapped protein 1 (IMP-1), microneme-1 protein (MIC1) and several rhoptry neck proteins (RON) which make the production of cost effective recombinant anticoccidial vaccines a more realistic prospect (Subramanian *et al*., 2008; Blake *et al*., 2011a; Jiang *et al*., 2012; Oakes *et al*., 2013; Yin *et al*., 2013). The success of such vaccines could be limited if there is pre-existing antigenic diversity.

Recombination at the sub-chromosomal level and mutation may give rise to novel genotypes which could become disseminated in *Eimeria* population in the field under natural condition, leading to the establishment of genetic variants of the parasites over time. An understanding of naturally occurring genetic diversity among field strains of *Eimeria*, especially at loci that are considered for inclusion as novel anticoccidial vaccines and are likely to be exposed to immune selection is an essential step towards the development of successful sub-unit vaccines.

### 1.3 Justification of the Research

The increasing interest in poultry production in Nigeria, evidenced by the proliferation of poultry farms, suggests an increased risk of outbreaks of coccidiosis. It is therefore pertinent to continually evaluate the dynamics of coccidiosis in this country so as to devise better control strategies for the disease.

Although different species of *Eimeria* have been reported in chickens in Nigeria, these reports were mainly based on traditional species identification using morphological and pathological criteria common in the diagnosis of coccidiosis (Bishu, 1982; Majaro, 1981; 1993; Muazu, *et al*., 2008; Jatau *et al.,* 2012). Traditional methods of identifying *Eimeria* species has the limitation of being subjective and may be unreliable in the diagnosis of natural infections (Long and Joyner, 1984; Woods *et al*., 2000a; Williams, 2001). This is because natural infections by *Eimeria* often occur as mixed infection with more than one species, potentially complicated by similar or overlapping morphological and pathological characteristics. Consequently, the use of molecular tools for accurate species identification and characterization of local isolates of this parasite is important.

Accurate identification of *Eimeria* species is important for proper diagnosis and disease control, also for studies of epidemiology and biology of the parasites, creation of new vaccines and selection of appropriate anticoccidial drugs (Tsuji *et al*., 1997; Woods *et al*., 2000a; Morris and Gasser, 2006; Sun *et al*., 2009; Lee *et al*., 2010).

More so, due to the species-specific nature of immunity in coccidia infection, live vaccines must contain each *Eimeria* species that is being targeted the cost of which significantly increases with every species that is included in the vaccine cocktail. It is therefore pertinent to identify the common *Eimeria* species that infect commercial chickens within a locality for effective and economical selection of live vaccines.

Vaccination with live parasites is effective but relatively expensive since it is highly labourious to produce, because it is only possible to generate oocysts using live chickens (Allen and Fetterer, 2002). There is also the risks of a negative effect on bird performance

due to intestinal damage caused by the live parasite, most notably a risk with wild-type vaccines (Waldenstedt *et al*., 1999; Williams *et al*., 1999; Allen *et al*., 2002). The search for a sustainable effective vaccine against coccidiosis has so far yielded the identification of novel recombinant vaccine candidates that confer significant immune protection against experimental challenges by the respective parasites (Blake *et al*., 2011a; Jiang *et al*., 2012).

However, with the established knowledge of the existence of antigenetic variation among strains of *Eimeria* parasites from different geographical locations of the world (Fitz-Coy, 1992; Martin *et al*., 1997; Danforth, 1998), there is need to understand the genetic diversity among the field *Eimeria* population from different parts of the world, especially at the loci that encode these vaccine candidates in order to validate the prospect of the proposed new vaccines providing global and long lasting control.

Whether drugs, vaccines or a combination prevail, it is clear that long-term control of coccidiosis will require identification of new targets within *Eimeria* parasites. This imperative underpins the need for genome sequencing of the most economically important *Eimeria* such as *E. tenella* because of its importance to agriculture and the research community. One of the significant benefits of *Eimeria* genome sequencing is that it increases opportunities for comparative genomics and the data will be of use to scientists with an interest in other apicomplexans, such as *Plasmodium* spp., *Neospora* and *Toxoplasma*.

To date the majority of research towards vaccine development and genome sequencing has been based on established archived laboratory strains from Europe and North

America and little is known about genetic diversity of *Eimeria* species in other regions, especially Africa. There is also no information describing the genetic diversity prevalent among Nigerian isolates of *Eimeria* species. More so, there is no work describing the coding and non-coding polymorphisms in the vaccine candidate genes of local isolates of *E. tenella*.

### 1.4 Aim of the Study

The aim of the study was to:

Determine the prevalence, identify the occurring species and the extent of genetic diversity of *Eimeria* species from commercial poultry farms in Zaria and environs, Kaduna state, Nigeria.

### 1.5 Objectives of the Study

The objectives of the study were to:

1. Determine the current prevalence of *Eimeria* infection in selected commercial poultry farms in Zaria and environs.
2. Carry out oocysts morphometric and molecular identification of *Eimeria* species occurring in the selected commercial poultry farms in Zaria and environs.
3. Conduct a molecular survey of the genetically variant *Eimeria* species of chickens (OTUs) in the selected commercial poultry farms in Zaria and environs.
4. Determine the extent of population genetic diversity within the internal transcribed spacer sequences 1 & 2 (ITS 1&2) of the *Eimeria* species isolated from Zaria and its environs.

5. Sequence a sub-unit vaccine candidate-encoding locus (AMA-1) from *E. tenella* isolates from Zaria and environs, to identify coding and non-coding polymorphisms.
6. Sequence the genome of one *E. tenella* isolate for identification of genome wide single nucleotide polymorphisms (SNPs) in comparison with existing reference strains.

### 1.6 Research Questions

1. What is the present prevalence of Coccidia infection in commercial poultry farms in Zaria and environs, Kaduna state, Nigeria?
2. What are the common *Eimeria* species occurring in commercial poultry farms in Zaria and environs, Kaduna state, Nigeria?
3. Are the genetically variant *Eimeria* species of chickens (OTUs) present in commercial poultry in Zaria and environs, Kaduna state, Nigeria?
4. What is the extent of genetic diversity among the *Eimeria* species occurring in commercial poultry farms in Zaria and environs, Kaduna state, Nigeria?
5. Is there genetic polymorphism among the AMA-1 coding sequence of different *Eimeria tenella* isolates from the study area and do they differ from that of isolates from different parts of the world?
6. What is the extent of genome wide genetic divergence of Nigerian *Eimeria tenella* isolates in comparison to the existing reference strains?

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction

Avian coccidiosis is an enteric protozoan disease of birds with greater economic importance in chicken than in other domesticated birds. It is caused by parasites from the genus *Eimeria* and seven species are widely recognized as the causative agents of the disease in chickens, of which *E. tenella, E. necatrix, E. maxima,* and *E. brunetti* are highly pathogenic, *E. acervulina* and *E. mitis* are less pathogenic, whilst *E. praecox* is regarded as the least pathogenic (McDougald, 2003; Shirley *et al*., 2005; Conway and McKenzie, 2007; Taylor *et al*., 2007).

Coccidiosis remains one of the major disease problems of poultry despite the advances made in prevention and control through chemotherapy, vaccination, management and nutrition (Graat *et al*., 1996). In all parts of the world where intensive rearing is practiced, coccidiosis represents a major disease problem demanding the attention of poultry producers, feed manufacturers, and poultry disease experts (Reid, 1978). The occurrence of clinical coccidiosis is directly related to the number of sporulated oocysts ingested by a bird at one time, the pathogenicity of the *Eimeria* species, the age of the infected chicken and the management system (Reid, 1990). In large population of chicken kept confined together, the risk of acquiring sufficient dose of oocysts is more likely to occur and the risk factor is very high for young chicken than older age groups, which develop immunity from pre-exposures (Shirley, 2005). Losses due to mortality following a severe outbreak may be devastating and incidence rates as high as 50% were observed to occur in the form of an outbreak in Nigeria (Abdu *et al*., 2008; Musa *et al*., 2010). However, losses due to morbidity may be even more costly without the producers being aware that

their flocks have any disease problem. In general, the losses caused by coccidiosis without including the sub-clinical coccidiosis are estimated to be 2 billion USD throughout the world (O'Lorcain *et al.,* 1996). Losses due to sub-clinical forms of the disease are heavy and can't be estimated (Haug *et al*., 2008a).

The extensive use of anticoccidial drugs has led to the development of resistant strains, which ultimately resulted in reduced activity of the drug against the agent (Reid, 1990; Graat *et al.,* 1996). The other approaches, alternative to drugs to minimize the losses due to coccidiosis are induction of protective immunity through vaccination and the use of naturally resistant birds (Pinard-van Der Laan *et al.,* 1998).

**2.2 Etiologic Agents of Avian Coccidiosis**

The coccidia of importance in domestic animals belong mainly to the genus *Eimeria*. The genus *Eimeria* is one of the 25 genera recognized under the Family *Eimeridídae* of the Subphylum Apicomlexa (Soulsby, 1982). There are seven recognized species of *Eimeria* infecting the chicken that are studied thoroughly, namely: *Eimeria acervulina*, *E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. These species are universally accepted and there are no doubts about their validity as regards the recent advances in molecular identification and typing of *Eimeria* species of chicken. However, there are two other species namely *Eimeria mivati* and *Eimeria hagani* that are also described in the literature but their existence is questioned by many researchers due to lack of valid molecular evidence for their existence (McDonald and Ballingall, 1983; Shirley *et al*., 1983). Therefore, these two species are often overlooked in publications that deal with species discrimination techniques (Fernandez *et al*., 2003; Morgan *et al*., 2009; Vrba *et al*., 2010).

*Eimeria mivati* was first described by Edgar and Seibold (1964) as a pathogenic Coccidium having oocyst morphology similar to *E. mitis*. However, subsequent cross-immunization study revealed that *E. mivati* is the same species as *E. mitis* (Shirley *et al*., 1983). Another study by Fitz-Coy *et al*. (1989) however, concluded that these are two distinct species based on the ultrastructure of their schizonts. Recently, the molecular characterization of strains of *E. mitis* by sequencing the 18S rRNA gene revealed two types of 18S gene which correspond to the previously published sequences of *E. mivati* and *E. mitis* a finding that further supports the fact that the two species are the same (Vrba *et al*., 2011).

With the recent development of more sophisticated molecular tools for the specific diagnosis and characterization of *Eimeria* infection, three genetic variants (operational taxonomic units OTU-X, OTU-Y and OTU-Z) genetically distinct from species of *Eimeria* identified previously in chicken have been described from Australian and African poultry farms (Morris *et al*., 2007; Cantacessi *et al* 2008; Fornace *et al*., 2013). Comparative analysis of these genetic variants with the established chicken *Eimeria* species have identified OTU-X as an *Eimeria maxima* population variant, while OTU-Y and OTU-Z were defined as cryptic strains or species most closely related to *Eimeria brunetti* and the haemorrhagic *Eimeria tenella*/*Eimeria necatrix* group respectively (Cantacessi *et al*., 2008).

### 2.3 Life Cycle of *Eimeria* Species

*Eimeria* exhibit a complex life cycle, comprising both endogenous and exogenous stages. The endogenous development process has asexual and sexual reproduction taking place

inside the gut epithelial cells of the bird, while the exogenous development involve maturation of the oocysts outside the bird by sporulation (McDougald, 2003).

The typical life cycle of *Eimeria* consists of three major phases namely sporogony, merogony (schizogony) and gametogony (Lillehoj and Trout, 1993; McDougald, 2003). During sporogony, which occurs outside the host under suitable environmental conditions (warmth, oxygen and moisture), four sporocysts each containing two sporozoites are formed within each oocyst (Williams, 1998; Allen and Fetterer, 2002).

Sporulated oocysts, when ingested by susceptible hosts, initiate the infective cycle (Williams, 1998; Allen and Fetterer, 2002). After ingestion excystation occurs, the released sporozoites invade the intestinal epithelium and round up to form a trophozoite followed by nuclear division to form an immature meront (schizont) by which the merogony stage commences. A varying numbers of merozoites are being produced asexually through multiple fission process by each meront. *E. tenella* has 2-3 generations of merogony (McDougald, 2003). The first generation schizont measures up to 54μm in diameter and may contain up to 900 first generation meroziotes. The mature schizont ruptures into the lumen of the crypts of the caecal glands 3 days post infection (PI) and the merozoites penetrate other epithelial cells to form young second generation schizonts. Colonies of the second generation schizonts mature by day 4 post infection (PI) and release about 300 second generation merozoites into the lumen of the cecum. When large numbers of second generation schizonts are involved, a massive haemorrhage into the caecal lumen may be evident at about day 4 PI (Soulsby, 1982). Second generation merozoites penetrate new epithelial cells and initiate either third generation of schizonts or the gametogonous cycle with the majority undertaking gametogony cycle.

Gametogony starts when merozoites invade cells and develop into either macrogamonts or microgamonts. The former gives rise to a single macrogamete whereas the latter undergoes multiple divisions resulting in the formation of numerous flagellated microgametes. Fertilization occurs when the microgamete invades cells containing macrogamete and a wall forms as the oocysts mature (Lillehoj and Trout, 1993; Conway and McKenzie, 2007). Although the general live cycle is the same for all *Eimeria*, host specificity, site of development, patent and prepatent periods and pathogenicity vary between species (Shirley, 1995). Only a small proportion of the millions of oocysts produced by a bird survives and become infective. Essential conditions for the survival are sufficient moisture, oxygen and suitable temperatures. Sporulation of *E. tenella* is most rapid at $28^0$C, slows down at 20 or $32^0$C and ceases at 8 or $37^0$C (Reid, 1978). Since oocysts are killed within 10 minutes of exposure at $55^0$C, heat furnishes a convenient laboratory method of sterilization. Similarly, oocysts are killed by freezing or even if a few ice crystals developed in suspension fluid (Reid, 1978). Oocyst survival time is greatly extended in the presence of high humidity. The optimum level of temperature and relative humidity required for sporulation of oocysts are $29^0$C and 50-75% respectively (Urquhart *et al*., 1987).

### 2.4 Transmission and Occurrence of Coccidia Infection

*Eimeria* parasite is transmitted via a resistant oocyst and infection occurs when a susceptible chicken ingests the sporulated oocyst from the environment (Shirley *et al*., 2005). Clinically infected and recovered adult birds may continue to shed oocysts in their faeces thereby contaminating the environment (Trees, 1999; David, 2000; Etuk *et al*., 2004). But birds with severe infection may die before oocysts have completed their development (Conway and McKenzie, 2007).

The wall of oocysts provides an effective protective barrier against the extremes of environmental conditions and mechanical disruption, as such *Eimeria* oocysts can be spread to poultry houses mechanically by many routes such as dust, boots, cloths, wheels, contaminated equipment and personnel who move between pens, houses or farms (Kiani *et al* 2007; Taylor *et al*., 2007). Flies, beetles, cockroaches, rodents, pets and wild birds have also been incriminated as mechanical vectors (Majaro, 2001). Sporulated oocysts may survive as long as 86 weeks in shaded soil (Sainsbury, 1992) and are resistant to most disinfectants (Guimaraes *et al*., 2007; Jatau *et al*., 2013; Samaha *et al*., 2013).

Incidence of outbreaks of coccidiosis is more common during the hot wet season than during cooler dry season. However, in intensively managed birds, outbreaks may occur at any season of the year (Adene and Oluleye, 2004; Etuk *et al*., 2004, Abdu, 2007). Prevalence rates of 33.3% and 12% of coccidiosis have been reported in different parts of Nigeria (Adene and Oluleye, 2004; Jatau *et al*., 2012). The use of cages for laying hens has greatly reduced the number of outbreaks that occur in these valuable birds (Reid, 1990). In cage system there is no contact between the birds and their faeces, thus transmission would be minimal. It was presumed that the possible causes of infection in cage birds are human beings and may be from feed processing manufacturers too. However, in deep litter poultry houses, which offer optimal condition of temperature and humidity for oocyst sporulation, the risk of infection is further increased (Urquhart *et al.*, 1987; Adene and Oluleye, 2004; Taylor *et al*., 2007; Jatau *et al*., 2012). Depending on the degree of vigilant control of the disease and the age of the chicken exposed to the risk, a prevalence rate as low as 6.8% and as high as 80.3% has been observed (Methusela, 2001).

## 2.5 Pathogenesis and Clinical Signs of Coccidiosis

Infection of chicken with single species of *Eimeria* is very rare under natural conditions, and mixed species infections are common (McDougald *et al*., 1986; Williams *et al*., 1996). Nevertheless, in many outbreaks the clinical entity can be ascribed principally to one species or occasionally a combination of two or three (Soulsby, 1986; Taylor *et al*., 2007). The clinical disease is dependent on the number of oocysts ingested by individual birds. If the environmental hygiene is poor, the number may be very large which is particularly true for *E. tenella* that have high biotic potential. But in very light doses no clinically recognizable symptoms may occur and thus, the morbidity and mortality increase in proportion to the size of the dose ingested (Soulsby, 1986; Taylor *et al*., 2007; Conway and McKenzie, 2007).

*Eimeria tenella* is one of the most common and pathogenic coccidia of domestic poultry, which is distributed worldwide. It develops in the caecum and adjacent areas of the digestive tracts and causes "bloody" or caecal coccidiosis. Because of the sudden onset of mortality in severe infections, this species has been the most feared by poultry producers (McDougald, 2003; Taylor *et al*., 2007). On flock basis the first sign of coccidiosis becomes noticeable at about 3 days after infection. The first sign of morbidity is a refusal to feed and drink. Chickens show droopiness, huddle to keep warm and passed out bloody diarrhoea. Blood loss from caecal lesions and characteristic odour may be noted shortly before mortality begins (McDougald, 2003; Taylor *et al*., 2007). The greatest haemorrhage occurs on the day 5 and 6 of infection, and by the day 8 or 9 the bird is either dead or on the way to recovery. Mortality is highest between the fourth and sixth day, death sometimes occurring unexpectedly due to excessive loss of blood (Soulsby, 1982; McDougald, 2003). Coccidiosis is a self-limiting disease. Regeneration

of the epithelium and glands is completed by the 10th day in light infections; healing in severe infections may take 3 weeks (Reid, 1978). In recovered birds, a chronic illness may develop as a result of a persistent caecal core. Red blood cells count and haematocrit value may decrease as much as 50% on the 5th and 6th day of infection. The normal weight is never fully regained during the growing period (Conway and McKenzie, 2007). Caecal coccidiosis due to *E. tenella* most frequently occurs in young birds especially those at 4 weeks of age. Older birds are generally immune as a result of previous infection (McDougald, 2003). Conway *et al*. (1993) reported mortality due to caecal coccidiosis of 5% and 25% at doses of $10^4$ and $10^5$ oocysts per bird respectively in 9 day-old male broiler chicks. Significant decrease in weight gain and feed conversion ratio were also observed at these doses. Lesion scores increased significantly with increasing oocyst doses but the distribution at the two highest doses ($10^4$ and $10^5$) were not significantly different from each other (Conway *et al*., 1993).

*Eimeria necatrix* is one of the most important pathogens of the small intestine of poultry, which is also distributed worldwide. The sexual development of the parasite occurs in the small intestine with the gametogonic cycle in the caecum (Soulsby, 1982). Massive dilation or "ballooning" of the middle intestine suggests the presence of *E. necatrix* coccidiosis although *E. maxima* may, also produce the similar lesion. Presence of schizonts which may be as large as 66 μm in diameter is diagnostic for *E. necatrix* (Conway and McKenzie, 2007). Infected birds show listlessness, drooping wings, humped back, and dehydration. Mortality begins on the 5th day, shows greatest severity on the $7^{th}$ day and extends to the 12th day. The principal lesions are found in the middle third of the small intestine and in severe cases marked congestion, bleeding and necrosis occur on the mucosal surface of the intestine. The appearance of the whitish or yellowish

plaques containing schizonts is the pathognomonic lesion. A focal type parasitic granuloma reaction is only induced by this species of coccidia (Reid, 1978). Despite its high pathogenicity, *E. necatrix* is poor in biotic potential (Soulsby, 1982).

*Eimeria acervulina* is very commonly seen worldwide in distribution and less pathogenic than the two aforementioned species. It is usually responsible for sub-acute or chronic intestinal coccidiosis in older birds and chickens at the point of lay. It mostly localizes and develops in the epithelial cells of the duodenum and jejunum (Soulsby, 1982). Within recent years especially in large poultry establishments, the significance of this species as a pathogen has increased steadily. The biotic potential is great, the prepatent and sporulation periods are short, and very large numbers of oocysts may accumulate in the environment. The severity of the disease depends directly on the numbers of oocysts to which the bird is exposed (Reid, 1978). The clinical signs consist of weight loss and watery whitish diarrhoea. At post-mortem, greyish white, pinpoint foci or transversely elongated "ladder like' areas are visible from the serosal surface of the duodenum (Conway and McKenzie, 2007).

*Eimeria maxima* is one of the commonest species in poultry and also has a worldwide distribution. Developmental stages occur in the middle small intestine, which shows haemorrhagic enteritis associated with thickening of the intestinal wall and some ballooning. Oocysts are large ovoid with distinct golden colour that is discernible by comparing oocysts of other species (Conway and McKenzie, 2007). This species should be classed as moderate to severely pathogenic. In severe cases, there is extreme emaciation, pallor, depigmentation of feathers and anorexia. Morbidity is readily measured by weight loss in artificial infections. Light infections may induce

depigmentation and poor carotenoid absorption with economic consequences where local preference brings premium prices for deep pigmentation (McDougald, 2003). Rapid development of immunity frequently aids in control of *E. maxima* coccidiosis.

*Eimeria brunetti* is uncommon, although markedly pathogenic when it does occur. Developmental stages are in the lower small intestine caecum and cloaca (Soulsby, 1982). In severe cases the lesions are characterized by coagulation necrosis which appears on days 4-7 producing a caseous eroded surface over the entire mucosa and the condition is typically a rectal coccidiosis. Haemorrhagic catarrhal exudates appear 4-5 days after experimental infection (McDougald, 2003). The dropping is fluid, may be blood stained and a mortality rate of 10% was from this species alone (McDougald, 2003).

### 2.6 Genetic Composition of *Eimeria* Species

In *Eimeria* species, three distinct DNA genomes have been defined namely the genome localized to the nucleus, the mitochondrion and the apicoplast organelle. In addition, a double stranded RNA genome associated with virus-like particles has often been found in these parasites (Lee and Fernando, 2000; Shirley, 2000; Han *et al*., 2011). The nuclear genomes of all avian *Eimeria* species investigated so far using Pulsed Field Gel Electrophoresis (PFGE) have been estimated to contain between ~50 and ~60 Mbp DNA (Shirley, 2000; Blake *et al*., 2011b). Likewise the complete mitochondrial genome sequences of the seven *Eimeria* species that infect the chicken have been assembled and are found to comprise ~6200 bp DNA with ~65% A+T content (Lin *et al*., 2011; Liu *et al*., 2012). This relatively high A+T content of the mitochondrion genome of *Eimeria* influences codon usage and, consequently, amino acid content of the three protein coding

genes *cox*-1, *cox*-3 and *cytB*. Sequencing of the larger plastid genome of *Eimeria tenella* identified ~35 kbp DNA with a similarly high A+T content (Cai *et al*., 2003). The double stranded RNA virus genomes have been identified within many isolates of chicken *Eimeria* species ranging in size from ~1.7 to >7.4 kbp. Hybridization studies on the double stranded RNA virus genomes suggest the existence of multiple, genetically distinct viral strains or species (Lee *et al*., 1996; Lee and Fernando, 2000).

The nuclear genomes of *Eimeria* spp. are haploid during the majority of the life cycle (Cornelissen *et al*., 1984). The genome of *Eimeria tenella* is the most extensively studied and it comprises between ~50 and ~60 Mbp DNA contained within 14 chromosomes that range in size between 1 and greater than 7 Mbp (Shirley, 1994). Most of the 14 chromosomes can be resolved individually and several, including the smaller chromosomes that can be most readily separated by PFGE (Sheriff *et al*., 2003) show polymorphisms in size (Shirley, 2000). The size polymorphisms appear to be highly stable as different populations of reference strains, passaged at regular intervals over many years, remain defined by chromosomes with the same characteristic sizes. For *E. tenella*, some small differences have also been observed between the molecular karyotypes of reference strains and lines characterised by phenotypic differences (Shirley, 1994), but the differences are small and have not been investigated further.

The development of a protocol based upon oocyst incubation in a hydrochloric acid/ethanol solution followed by multiple freeze–thaw cycling has permitted the release and spread of intact chromosomes, confirming previous PFGE based estimates of a 14 chromosome karyotype for all *Eimeria* species that have been investigated (Shirley *et al*., 1990; Shirley, 1994a; del Cacho *et al*., 2005). Comparison between *E. tenella* strains has

revealed variable sizes for chromosomes 1–4 and 11 (Shirley, 2000; Sheriff *et al*., 2003). Variation by as much as 5% for chromosome 1 between extreme examples has been used as a genetic marker in linkage analyses (Shirley and Harvey, 2000). The molecular basis for the observed polymorphism remains unclear, although the highly repetitive nature of the *E. tenella* genome is likely to encourage non-homologous genetic recombination, resulting in length polymorphisms (Shirley *et al*., 1990).

The molecular karyotypes of other avian species of *Eimeria* appear similar to that of *E. tenella* (Shirley *et al*., 1990; Fernando and Pasternak, 1991; Shirley, 1997) with respect to both the numbers of chromosomes and their sizes. Each *Eimeria* genome is represented by a series of chromosomes including telomeres and centromeres (del Cacho *et al*., 2005). Transmission electron microscopy studies have revealed a constant telomere length of 32 nm for *E. tenella* and a consistent centromere index per chromosome among several strains, although figures vary between chromosomes, thereby providing distinctive identifiers (del Cacho *et al*., 2005). Intronic sequences are common within most coding regions and there is proteomic evidence for alternative splicing (Lal *et al*., 2009). Unusually, among apicomplexan parasites, sequences related to eukaryotic transposable elements are readily identified. It has been suggested that these are most similar to non-LTR LINE-like retrotransposons (long terminal repeat and long interspersed nuclear elements) (Ling *et al*., 2007). Most strikingly, the sequencing and assembly of *E. tenella* chromosome 1 revealed an unusual segmented structure to the chromosome, which contains three repeat-rich segments flanked by four repeat-poor segments (GenBank Accession number AM269894). The repeat-rich segments contain large numbers of simple sequence repeats and LINE-like elements as well as highly variable A+T content, C+G to G+C ratio and second-order Markov entropy, prompting the identifier ‘feature-

rich' (R-segment) (Ling *et al*., 2007). In contrast, the repeat-poor segments exhibited less variation in all measures, prompting the identifier 'feature-poor' (P-segment). Whole genome HAPPY mapping now suggests that the segmental organization described from chromosome 1 is also present throughout the rest of the *E. tenella* genome (Lim *et al*., 2012). Preliminary characterization of predicted gene structures within the R- and P-segments suggests shorter coding sequences with larger exons and less numerous introns in the latter. Restriction fragment length polymorphism (RFLP) comparison of *E. tenella* genomic DNA revealed polymorphism between the Houghton, Weybridge and Wisconsin strains for all four of the R-segment probes tested, but none of the four P-segment probes, suggesting a higher rate of genome evolution within the feature-rich regions. Comparative studies with the *E. maxima* genome suggests that R and P regions are also present throughout the genome of this species (Blake *et al*., 2011b).

Eimerian genes are known to feature large numbers of repetitive DNA sequences ( Jenkins, 1988) and early reports of numerous trinucleotide GCA repeats (and alternative frame permutations CAG, AGC, TGC, GCT and CTG) are supported by more systematic studies of published EST data and the *E. tenella* chromosome 1 assembly. Almost 3000 simple repeat units were identified on chromosome 1 and more than 60% of these were GCA-based (Ling *et al*., 2007). Other common repeats included a telomere-like heptamer AGGGTTT, representing nearly 20% of the repeats on chromosome 1, and the palindromic octamer TGCATGCA, which has been described previously within several other apicomplexan genomes (Chapman *et al*., 2013). These simple sequence repeats were confined largely to the R-segments in the *E. tenella* chromosome 1 assembly, where triplet repeats and AGGGTTT represent ~14% of the sequence (Chapman *et al*., 2013). Both EST and genomic analyses identified frequent triplet repeats in putative coding

regions, a feature confirmed also by proteomic analysis (Lal *et al*., 2009; Ling *et al*., 2007; Shirley, 2000). Importantly, triplet repeats do not interfere with the coding frame in expressed sequences, unlike heptamer and octomer repeats (Chapman *et al*., 2013). To date, no functional association has been made with these translated repetitive sequences, although they have been hypothesized to play a role in genome evolution and diversification as possible hotspots of recombination (Ling *et al*., 2007).

Other repetitive sequences include multiple putative transposable sequences and arrays of tandemly repeated 5S and 18S–5.8S–28S ribosomal genes (Chapman *et al*., 2013). The high copy number of the rDNA arrays and the ITS regions (ITS-1 and ITS-2) has promoted their use as targets for molecular diagnostics and phylogenetic analyses, although it is important to note the existence of polymorphism between copies within a single genome (Blake *et al*., 2006; Vrba *et al*., 2011).

## 2.7 Diagnosis of Coccidia Infection

### 2.7.1 Traditional parasitological diagnosis

Diagnosis of coccidiosis in poultry flocks continues to rely on necropsy and the examination of birds for intestinal lesions in different areas of the gut. Because of the site-specificity of invasion, the presence of lesions can provide insight into which species of coccidia is/are responsible for clinical symptoms (Long and Joyner, 1984; Conway and McKenzie, 2007). Diagnosis may be corroborated by microscopic analysis of shape and size of *Eimeria* oocysts shed in faeces from infected birds. Additional criteria classically used to characterize *Eimeria* species include pre-patent period, minimum sporulation time, tissue location of parasitic forms and immunological specificity (McDougald, 2003). However, definitive identification of a particular *Eimeria* species

based on morphological and pathological criteria can be tedious, requires highly qualified personnel and may be confounded by the overlapping features observed in different *Eimeria* species (Long and Joyner, 1984).

*2.7.1.1 Gross lesions*

The procedures for sacrificing birds and post-mortem examination are based on the technique discussed by Zander (1978). The entire length of the external serosal surface of the digestive tract from the gizzard to the lower rectum needs to be examined under strong light. In examining the serosal surface a search should be made for whitish plaques or petechiae. Whitish streaks or rounded colonies of oocysts in the duodenal area are often indicative of *E. acervulina* or *E. mivati*. In the mid gut area on both sides of the yolk sac diverticulum, whitish plaques may be produced by colonies of *E. necatrix* schizonts (Conway and McKenzie, 2007). While cutting, watch for thickened areas indicating parasitic invasion of the mucosa or submucosa. Presence of mucus, blood, casts, or cores and presence of cheesy coagulation necrosis should be noted. Presence of blood in the caeca suggests a diagnosis of *E. tenella*. But bleeding may originate from the more anterior zones of the intestine and moving to the caecum may lead to a misdiagnosis of *E. necatrix* as *E. tenella* infection. As differential diagnosis histomoniasis, Haemorrhagic Syndrome and ulcerative and necrotic enteritis may also produce somewhat similar gross lesions (Reid, 1978).

*2.7.1.2 Microscopic examinations*

Parasitic stages demonstrated in fresh smear preparations are usually adequate in confirming a diagnosis. A standard parasitological technique for demonstration of the parasite from intestinal mucosa scraping can be used efficiently (Conway and McKenzie,

2007). However if no suspicious lesions have been noted on the intestine, select and take scrapings from the following parts of the intestine:

The duodenal loop below the entrance of the bile duct.

The mid gut (jejunal) region near the yolk sac diverticulum (Michele's diverticulum).

The lower intestinal area just above the union of caecal pouches and

An area from the middle of the cecum

The rectal area (Conway and McKenzie, 2007).

For example, presence of distinctive schizonts of *E. necatrix* from the mid gut area is pathognomonic for this species (Conway and McKenzie, 2007).

Oocysts are the most commonly recognized stages of the lifecycle and may be present in large numbers. However, because they develop late in the cycle they may be entirely missing in the presence of severe coccidiosis. Oocyst size and shape are less useful as diagnostic characteristics in chickens than in many other animals, because they are similar except with *E. maxima*. Measurement of a number of oocysts (10 suggested) using an ocular micrometre is required (Conway and McKenzie, 2007). Length and width must be determined by selecting a side and not an end view of the oocyst. Thick faecal smears frequently prevent securing the necessary side view of the oocyst. Average length, average width, range in length, and range in width should be determined (Reid, 1978). Oocysts shape differs from species to species. Reid, (1978) described the oocysts of *E. tenella, E. maxima, E .acervulina* and *E. brunetti* as being ovoid; *E. necatrix* as oblong ovoid; and "*E. hagani"* as broadly ovoid. However, since these descriptive terms are subjective, "shape index" determined by dividing the mean length by the mean width may be more useful (Conway and McKenzie, 2007).

In critical studies on species identification, additional techniques other than microscopic examinations are needed such as:

• Time required for sporulation and prepatent period

• Cross-immunity tests

• Location of the parasite in relation to the host epithelial cells nuclei and

• Tissue culture or egg embryo developmental differences may prove useful in identification of some species (Reid, 1978).

### 2.7.2 Molecular approach in diagnosis and characterization of coccidia parasite

Traditional methods of identification of *Eimeria* species has the limitation of being subjective and may be unreliable in the diagnosis of natural infections (Long and Joyner, 1984; Woods *et al*., 2000a; Williams, 2001). This is because natural infections by *Eimeria* often occur as mixed with more than one species, which might be having similar or overlapping morphological and pathological characteristics. Some of the limitations of the traditional methods have been overcome by the development of various biochemical and molecular methods.

#### *2.7.2.1 Multilocus enzyme electrophoresis (MEE)*

The principle of multilocus enzyme electrophoresis (MEE) is that non-denatured proteins (e.g., enzymes) may be separated by their migration through a gel under the influence of an electric field (Andrews and Chilton, 1999). Homologous enzymes may differ in charge and size, due to underlying variation in the originally transcribed DNA sequence. As the rate of a protein's migration through a gel under an electric field also varies with charge and size, enzymes from different individuals or species may be separated. Their relative positions can then be revealed by appropriate enzyme specific staining (Andrews and

Chilton, 1999). The equipment and specific stains necessary for MEE are in common use, and this technique has been successfully applied to investigate genetic variation within and among species of *Eimeria* infecting chickens where each of the species examined could be identified and differentiated using four distinct enzymes (Shirley, 1975). Multilocus enzyme electrophoresis was also shown to be effective for the analysis of mixed field samples provided each species constitute a minimum of 10–20% of the oocysts in the original sample (Chapman, 1982). Numerous laboratories have since utilized MEE as a means to aid in the differentiation among *Eimeria* species. This approach has been shown to be effective for samples taken from around the world (Shirley *et al*., 1989), including Australia (Andrews *et al*., 1990), Canada and the U.S.A (Johnston and Fernando, 1997), Japan (Nakamura *et al*., 1986), and Sweden (Thebo *et al*., 1998).

Compared with other organisms, Coccidia are well suited to this technique; large numbers of oocysts can be collected and pooled from faeces, and, if needed, they can be passaged through chickens to amplify oocysts from an initial small sample (Long and Joyner, 1984). However, there are some significant limitations. Large number of oocysts may not always be available, and passaging adds another expense and delays the test result (Chapman, 1982; Procunier *et al*., 1993; Fernandez *et al*., 2003b). The process is awkward and time-consuming (Procunier *et al*., 1993; Fernandez *et al*., 2003b). There is strong selection pressure for stability in protein function, resulting in relatively limited variation in a protein's amino acid sequence; as MEE detects such variation, its application is restricted because of the limited magnitude of variation present (Shirley, 1994a; Fernandez *et al*., 2003a;b). In addition, even if there is underlying variation in the

DNA sequence, this may not translate into a variation in amino acids, and even if it does, this may not result in varying mobilities during electrophoresis (Shirley, 1994a).

*2.7.2.2 Southern blot analysis*

Southern (1975) developed a method for the detection of specific fragments of DNA separated by electrophoresis. Genomic DNA is digested using one or more restriction endonucleases. The resultant fragments are separated on a gel by electrophoresis, blotted to a solid support, hybridized to one or more specific probes, and their positions detected by autoradiography or fluorography. This method's reproducibility is based on the ability of restriction enzymes to cleave DNA at specific recognition sites in a nucleotide sequence. For example, for the nucleotide sequence 5′- GGATCC-3′, the restriction enzyme BamH1 will invariably cut the DNA strand between the two guanine bases. Southern blot hybridization coupled to restriction fragment length polymorphism analysis, RFLP was first applied to *Eimeria* species of chickens by Ellis and Bumstead (1990). Genomic DNA of *E. tenella, E. acervulina* or *E. necatrix* was digested with the endonuclease EcoRI and probed with an RNA probe (which was previously prepared from total cellular RNA extracted from sporulated oocysts). The three species could be readily differentiated. Restriction fragment analysis using the endonuclease BamH1 and a pRibI probe could not distinguish among four different strains of *E. tenella* (Houghton, Weybridge, Wisconsin and Doran strains) (Ellis and Bumstead, 1990). However, a more recent study did reveal variation within this species. Shirley (1994a), examined seven strains of *E. tenella* using a panel of endonucleases (including HinfI, RsaI, HpaII and EcoRI). Four probes were derived from repetitive sequences of genomic DNA of *E. tenella* and used to hybridize to the restriction fragments produced via separate digestions with the endonucleases. Variation was detected among strains of *E. tenella*, including the

Houghton and Wisconsin strains, for which no variation was previously detected (Ellis and Bumstead, 1990). The detection of such intra-specific variation suggested that Southern blot analysis was more sensitive than MEE (Shirley, 1994a). While the technique employs common reagents, an appropriate probe must be designed to hybridize specifically to the digested DNA fragments. Other limitations include the complexity (involving more steps than allozyme electrophoresis), and the need for relatively large numbers of oocysts to process (Procunier *et al*., 1993; Shirley, 1994a; Fernandez *et al*., 2003b).

*2.7.2.3 Pulsed-field gel electrophoresis (PFGE)*

Traditional methods of gel electrophoresis have a limited ability to separate DNA molecules of above a certain threshold size (greater than 15 to 20 kilobases) (Carle *et al*., 1986). These large molecules tend to migrate at the same rate, regardless of size. However, by making minor alterations to the electrophoretic protocol, DNA molecules of 50 kbp to several Mbp can be separated. One method is to use 'pulsed-field gel electrophoresis' (PFGE), where an electric current is applied intermittently across the gel encouraging migration of large molecules. The position of the DNA molecules is then detected by staining with ethidium bromide or by hybridization to a radioactive probe (Fernando and Pasternak, 1991; Shirley, 1994b).

The chromosomes of *Eimeria* fall within the appropriate size for analysis by PFGE, and have been investigated. Shirley *et al*. (1990) used PFGE to characterize the karyotype of *E. tenella*. While it had been suggested that the haploid *E. tenella* nuclear genome had 5 chromosomes, PFGE revealed at least 12. A subsequent study (Shirley, 1994b) also revealed differences in the karyotype within *E. tenella*, uncovering chromosomal size

variation between strains. In addition, particular probes were found to bind to some individual chromosomes, but not others, revealing basic sequence differences between homologous pairs.

Pulsed-field gel electrophoresis provide broad information about the genome, such as chromosome size and minimum number. Additional information can be obtained from the differential binding to DNA probes of chromosomes previously separated by PFGE (Shirley, 1994b). However, it does not provide detailed information about the genome and is very time-consuming to carry out (Shirley, 1994b).

*2.7.2.4 Polymerase chain reaction (PCR)-coupled methods*

A limitation of the molecular methods described earlier is that relatively large amounts of parasite material are required for effective analysis and/or detection. In some cases, this may require the propagation of the parasite(s) ('passaging') in live chickens to produce sufficient numbers of oocysts, which can be time-consuming, laborious and costly. Clearly, the advent of the polymerase chain reaction (PCR) (Saiki *et al*., 1985, 1988) overcame this limitation and has enabled the analysis of tiny amounts of parasite material. The PCR enables the selective amplification of DNA from complex genomes.

The principle of the PCR technique is that the double-stranded genomic DNA template is denatured by heating, and the temperature is then decreased to allow one or more oligonucleotide primers to hybridize (anneal) to their complementary sequences on opposite strands of the template; the template-directed DNA synthesis (extension) then proceeds in both orientations from the primer sites by enzymatic catalysis with a thermo stable DNA polymerase and results in the production of double-stranded products. This

synthesis is usually repeated 20–40 times in an automated thermal cycler. In each cycle, the template is replicated by a factor of two, such that, upon completion of the cycling, millions of copies of the DNA target are available for subsequent analyses. Many different PCR approaches are available (Monis *et al*., 2005; Gasser, 2006), and those applied to *Eimeria* are reviewed in the following sections.

*2.7.2.4.1 Amplified fragment length polymorphism (AFLP)*

The method of AFLP is a high stringency fingerprinting technique which relies on the selective amplification of restriction fragments produced from genomic DNA and their subsequent display by denaturing gel electrophoretic analysis (Vos *et al*., 1995). There are three main steps in the process: (a) total digestion of genomic DNA and ligation of oligonucleotide adapters to the resultant fragments; (b) selective amplification of certain sets of restriction fragments; and (c) analysis by gel electrophoresis of the amplified fragments (Vos *et al*., 1995). The PCR amplification of step (b) is made possible by using the adapter and restriction site sequence as the target site for primer annealing. This process is made selective by the choice of primers which extend into the restriction fragments; only those fragments in which primer extensions match the nucleotides flanking the restriction site will be amplified (Vos *et al*., 1995). Typically, between 50 and 100 fragments are amplified, and these are then separated by gel electrophoresis to create a 'fingerprint'. This useful technique, while using highly specific primer annealing and amplification, does not require previous knowledge of the genome sequence (i.e., primers do not need to be specifically designed to target the genome of the organism of interest). AFLP has been used to derive polymorphic markers for *E. tenella* for the construction of a genetic linkage map (Shirley and Harvey, 2000). Different combinations of primers were used on the same source material with varying degrees of

success; the best combinations yielded 10 polymorphic fragments per gel (Shirley and Harvey, 2000). Blake *et al*. (2003) examined a single (designated Houghton) strain of *E. maxima* using a panel of nine *E. maxima* samples and a range of primers, reproducible AFLP fingerprints were consistently generated. Various combinations of restriction enzymes and primers should be evaluated to optimize the conditions and number of amplified fragments generated; also high quality and high molecular weight DNA is required. As the primers employed are not designed to specifically target the organism under investigation, the technique can give spurious results if contamination is present in the sample (Blake *et al*., 2003). Also, this technique cannot be used to detect mixed-species infections, as multiple sequences can be hidden within bands of the same molecular weight on electrophoretic gels.

*2.7.2.4.2 Random amplification of polymorphic DNA (RAPD) and sequence-characterized amplified regions (SCARs).*

The random amplification of polymorphic DNA (RAPD) or arbitrarily primed-polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990; Williams *et al*., 1990) relies on the amplification of genomic DNA fragments using (usually) single primers (10-mers) of arbitrary sequence, and subsequent electrophoretic separation of the amplicons. The advantages of RAPD include speed, simplicity, the relatively tiny amounts of genomic DNA required and the ability to screen the entire genome. Although RAPD is a useful method, there may be problems with specificity and/or reproducibility of results due to the low annealing temperatures (i.e., 35–45 °C) frequently used in the PCR (MacPherson and Gajadhar, 1993). The RAPD technique has been used to study variation within species of *Eimeria* from chickens. Shirley and Bumstead (1994) examined seven strains of *E. tenella* of varying biological phenotypes. Twenty-four arbitrary primers were used in separate PCRs to amplify DNA. Amplicons were

electrophoretically separated on agarose gels to display profiles. Of 199 bands displayed, 4 (2%) were considered to be variable among the seven samples examined, providing strain-specific markers (Shirley and Bumstead, 1994). Barta *et al*. (1998) employed RAPD to confirm the identity of five strains of *E. maxima*. However, with the seven arbitrary primers tested, these authors were not able to identify sufficient unique bands to discern the relationships among the strains. Some researchers have used the calculation of similarity coefficients to gain a measurement of degree of relatedness (Procunier *et al*., 1993; Johnston and Fernando, 1995; Costa *et al*., 2001). The similarity coefficients depend on the number of bands amplified from each of a pair of species/strains and the number of shared bands (Procunier *et al*., 1993; Johnston and Fernando, 1995). Another use of RAPDs is for the identification of, and discrimination among, species. The typical approach has been to test numerous random primers and establish which ones result in unique patterns for each species. MacPherson and Gajadhar (1993) were the first to use this technique to distinguish among seven species of *Eimeria*. Another early study by Procunier *et al*. (1993) applied nine primers to six species of chicken *Eimeria*; this approach could be used for the differentiation among all species tested. More recently, studies have confirmed the ability of RAPD techniques to distinguish between *Eimeria* species from chickens (Johnston and Fernando, 1995; Tsuji *et al*., 1997; Costa *et al*., 2001; Fernandez *et al*., 2003a). As RAPD is usually conducted under low-stringency conditions (i.e., low annealing temperature), there can be problems with the specificity and reproducibility of results (Fernandez *et al*., 2003a; b). Also, this technique is inappropriate for detecting mixed-species infections, as multiple sequences can be hidden within co-migrating bands on an electrophoretic gel (Fernandez *et al*., 2003b). The main advantage of RAPD over other PCR-based techniques is that primers are arbitrary in sequence, requiring no previous knowledge of the genome sequence (s) (MacPherson

and Gajadhar, 1993; Procunier *et al*., 1993; Johnston and Fernando, 1995; Fernandez *et al*., 2003b). The technique is also rapid and relatively simple, and requires relatively little genomic DNA template (MacPherson and Gajadhar, 1993; Johnston and Fernando, 1995).

A further advantage of RAPD is that it can be used to define 'sequence-characterized amplified regions' (SCARs), which can represent species-specific or strain-specific genetic markers. RAPD bands displayed in an electrophoretic gel are excised, purified, cloned and sequenced. Once the sequences have been determined, they are called SCARs. Primers designed to target these sequences can be used for targeted PCR-amplification (at high stringency) from species or strains. Recently, this approach has been applied to *Eimeria* of chickens (Fernandez *et al*., 2003a; b). Species-specific SCARs were defined for each of the seven species of *Eimeria* and a primer set was designed for each (Fernandez *et al*., 2003a). Each of these primer sets reliably and reproducibly amplified DNA from the appropriate species, resulting in a PCR product that appeared as a single band on agarose gel, but not from heterologous species (Fernandez *et al*., 2003a). In a subsequent study (Fernandez *et al*., 2003b), the same research group developed a multiplex PCR assay based on SCAR markers. The development of a multiplex PCR for the simultaneous detection of all seven species of *Eimeria* from chickens has been a very valuable technological advance in diagnosis (Fernandez *et al*., 2003b).

*2.7.2.4.3 Polymerase chain reaction (PCR)-based detection methods using genetic markers in nuclear ribosomal DNA*

In contrast to fingerprinting methods, PCR detection assays rely on the amplification of particular regions of genomic DNA using a defined set of oligonucleotide primers. Stucki

*et al*. (1993) designed a primer pair to amplify by PCR the 5S ribosomal RNA unit of *E. tenella*. While this PCR could amplify from as few as two oocysts of *E. tenella*, no product was amplified from any of the five other *Eimeria* species tested. Similarly, Molloy *et al*. (1998) designed primers to a sporozoite antigen gene. The assay was specific for *E. acervulina* and did not amplify product from any of the other six *Eimeria* species from chickens. In both cases, an effective tool was developed for the identification of a single species of *Eimeria*, but the approach had a limitation in that heterologous species could not be detected.

Other, more recent studies focused on the use of the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA (rDNA), which separate the ribosomal genes. While the ribosomal genes themselves tend to be conserved, these spacers are relatively heterogeneous in length and sequence among species, such that specific primers may be designed to the flanking gene sequences. Like other regions of nuclear rDNA, these spacers evolve in a concerted fashion and are repetitive in nature (Elder and Turner, 1995), making any PCR assay (based on their use) sensitive. Schnitzler *et al*. (1998, 1999) chose the ITS-1 as a target for PCR. Initially, genus-specific primers to conserved regions flanking the ITS-1 were used to amplify a product from each of the seven species of *Eimeria*. The amplicon from each species was then purified, cloned and sequenced. Seven different primer pairs were designed to unique sections of the ITS-1 sequence, one for each species. Other workers (Lew *et al*., 2003; Su *et al*., 2003) used a similar approach, employing genus-specific primers as a starting point, cloning and sequencing the ITS-1 region, and then designing species-specific primers. The above studies employed one of two methods for the detection of the amplicons produced. Typically, PCR products underwent electrophoresis on ethidium

bromide stained agarose gels, their sizes being displayed (Schnitzler *et al*., 1998; Lew *et al*., 2003; Su *et al*., 2003). While agarose gels allow the resolution of a PCR product based on size, they are limited in their ability to display sequence variation among amplicons, unless it relates to a considerable length difference. In these studies (Schnitzler *et al*., 1998; Lew *et al*., 2003; Su *et al*., 2003), each primer pair amplified specifically and reproducibly from the appropriate species but not from heterologous species. However, no evaluation of all primers in a single, multiplex reaction was reported. Hence, in using these PCR-based assays, each sample must be subjected to multiple PCR reactions to detect which species are present, a process that is labourious and time consuming. If a single set of primers could simultaneously amplify from each of the seven species of *Eimeria*, producing specific amplicons which are clearly distinguishable in size among all species, then much labour could be avoided and the assay would be less expensive to perform. In addition, the electrophoretic methods used are unable to detect sequence variability in ITS-1 amplicons.

*2.7.2.4.4 Polymerase chain reaction (PCR)-coupled manual and semi-automated electrophoretic methods.*

The direct sequencing of PCR ITS-1 or ITS-2 amplicons of *Eimeria* species of chickens is not feasible due to sequence heterogeneity within individual isolates (Schnitzler *et al*., 1998; 1999; Woods *et al*., 2000a; b; Lew *et al*., 2003). Thus, sequencing of cloned amplicons has been the most commonly used approach to define species specific genetic markers in these spacers. However, this approach has limitations in that it does not allow an accurate characterization of the sequence variability and because PCR-induced artefacts may lead to erroneous sequence data. Recently, electrophoretic approaches have been utilized for the direct display of sequence heterogeneity within and among amplicons (Gasser, 2006) and have been applied to the internal transcribed spacers of

*Eimeria*. Woods *et al*. (2000b) employed PCR based RFLP analysis on high-resolution electrophoretic gels for the initial characterization of sequence variation in the ITS-2 within and among samples representing six different species of *Eimeria* from chickens from Australia, in order to assess whether this rDNA region would provide genetic markers for their specific identification. The ITS-2 was amplified from genomic DNA by PCR using a radiolabelled primer set, the forward primer (WW2) being *Eimeria*-specific and the reverse one (WW4R) being Eimeriidae-specific, digested separately with three restriction endonucleases (CfoI, Sau3AI or TaqI) and the fragments separated by denaturing gel electrophoresis. The PCR products amplified separately from isolates representing six species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox* and *E. tenella*) varied in size from ~370 to 620 bp on agarose gels, with differences in size and number of bands among species but limited variation within a species. The PCR-RFLP analysis of ITS-2 amplicons on denaturing gels gave characteristic profiles for individual species (except for minor variation in profiles within some species), which indicated that ITS-2 contained useful genetic markers for the identification of six *Eimeria* species occurring in Australia. Subsequently, Woods *et al*. (2000a) employed denaturing gel electrophoresis and single strand conformation polymorphism (SSCP) for the direct analysis of radiolabelled ITS-1 and ITS-2 amplicons from *Eimeria* species from chickens. While the former electrophoretic approach separates single-stranded molecules based on size alone, the latter has the major advantage that it separates them based on both size and sequence (Gasser and Zhu, 1999). Both electrophoretic approaches allowed the unequivocal identification and delineation of all six species tested; in addition, the SSCP allowed the detection of population variation within *E. acervulina* not detectable by denaturing gel electrophoresis (Woods *et al*., 2000a).

Previous studies (Woods *et al*., 2000a; b) were extended to establish semi-automated electrophoretic approaches for the specific identification and differentiation of all seven currently recognized species of *Eimeria* infecting chickens (Gasser *et al*., 2001, 2005). The ITS-2 was PCR-amplified from all seven species using the original set of oligonucleotide primers (WW2 and WW4R), of which WW2 was labelled fluorescently. Following the PCR, the amplicons were diluted (1/30) in a specific (denaturing) loading buffer, heat-denatured and subjected directly to slab gel (Gasser *et al*., 2001) or capillary electrophoresis (CE) (Gasser *et al*., 2005). The chromatograms captured were stored electronically and then analysed using a specific software program. Using well-defined control DNA samples representing monospecific lines of *Eimeria*, particular peaks in the chromatograms were defined for the identification of each of the seven species and for their unequivocal differentiation. Some variation in the positions of 'minor' peaks in the chromatograms (reflecting population variation) was detectable for some species, but did not interfere with specific identification (Morris and Gasser, 2006).

Clearly, the CE approach has major advantages over manual electrophoretic techniques, mainly in relation to electrophoresis time, sample through-put, and data storage and analysis capacities. There is no need for the use of ethidium bromide or radionucleotides, or the pouring, handling and/or exposure of electrophoretic gels, thus substantially reducing overall time and cost. Other advantages are that chromatograms representing different samples, run on different gels and different days, can be scored automatically and stored electronically in a spreadsheet format, and can be retrieved at any time point for comparative analyses. The CE approach is thus suited for the screening of large numbers of samples and, although qualitative, is currently less expensive to carry out

than, for example, real-time PCR, and has the added advantages of being able to detect genetic variability (Morris and Gasser, 2006).

## 2.8 Immunity

Research work focussing on the understanding of the immunobiology of coccidiosis is dated as far back as 6 decades and by the end of 1962, it was known that: (a) a single infection with various species of *Eimeria* confers solid resistance to re-infection and this is increased further after a second infection (Rose and Long, 1962); (b) immunity is expressed against the very early asexual stages of infection such that, although penetration of epithelial cells by sporozoites may occur, subsequent development is blocked (Pierce *et al*., 1962); (c) immunity is not confined to early stages of parasite development but also affects later stage merozoites and sexual stages (Rose, 1963); (d) in general, immunity to one species confers no protection against other species (Rose and Long, 1962), though it was later shown that some degree of cross-protection can occur between closely related pairs of *Eimeria*, such as *E. maxima* and *E. brunetti* (Rose, 1967a); *E. tenella* and *E. necatrix* (Rose, 1967b) and (e) B cells and the antibodies they produce play little, if any role in resistance to re-infection, implicating cell-mediated responses in acquired immunity (Long and Pierce, 1963). Immunity to *Eimeria* is complex, multifactorial and influenced by host and parasite, with different elements playing greater or lesser roles in three different types or stages of immunity: innate resistance to primary infection; acquired immunity to reinfection and maternal immunity (Rose, 1970).

Much of the understanding of immune response to coccidia has come from work on model infections with *E. vermiformis* in genetically altered mice (Smith and Hayday,

1998), where it was observed that antigen specific T-cell receptor (TCRγδ) $CD4^+$ major histocompatibility complex type II (MHC-II)-restricted T cells that produce gamma interferon (IFN-γ) to be essential for antigen specific resistance to both primary and secondary infections. Additionally, B cells and interleukin-6 (IL-6) are minor but necessary components of resistance to primary infections (Smith and Hayday, 1998). Other, non-class II-restricted cells appear to be involved in effecting good "memory" responses to challenge infections.

Chicken intestinal lymphocytes have been phenotyped using monoclonal antibodies, and found to exhibit markers homologous to those of murine and human CD3, CD4, and CD8 T cells (Chan *et al*., 1988; Chen *et al*., 1988; Tomley *et al*., 1993; Lillehoj, 1994; Lillehoj and Trout, 1996). The CD3 polypeptide complex is found in association with the antigen-specific T-cell receptor (TCR) (Bucy *et al*., 1988) characterized by αβ or γδ heterodimers (Chen *et al*., 1988). The $CD4^+$ T (helper) cells recognize MHC-II antigens, and $CD8^+$ T (cytotoxic/suppressor) cells recognize MHC-I antigens (Lillehoj and Trout, 1996).

In chickens, TCR $\gamma\delta^+$ T cells can be $CD4^+$ or $CD8^+$ (Lillehoj and Trout, 1996). The roles of these T cells in response to avian *Eimeria* infections appear somewhat different from those in murine *Eimeria* infections. Studies in which these T-cell populations in chickens infected with *E. acervulina* or *E. tenella* have been selectively depleted by treatment with monoclonal antibody (Trout and Lillehoj, 1996) show a different contribution of $CD4^+$ and $CD8^+$ T cells to resistance and immunity, which appears, as judged by oocyst output, to be dependent on the infecting species of coccidia. In chickens infected with *E. acervulina*, $CD4^+$ T-cell depletion did not significantly increase oocyst production during either primary or challenge infection (Trout and Lillehoj, 1996). Additionally, fewer

oocysts were produced during challenge than during primary infection. These results suggest that $CD4^+$ T cells are not significant effectors of resistance or immunity to *E. acervulina*. On the other hand, depletion of $CD4^+$ cells in *E. tenella* infections resulted in increased oocyst output during primary but not challenge infection, indicating that $CD4^+$ cells are important effectors of resistance to primary *E. tenella* infections. Depletion of $CD8^+$ T cells significantly decreased oocyst output during primary infections of both *E. acervulina* and *E. tenella*. It is theorized that this effect is due to a reduction in the number of $CD8^+$ cells that serve as transporters for sporozoites. However, $CD8^+$ cell depletion results in a larger oocyst output after a challenge infection. These results indicate $CD8^+$ cells may not be effectors of resistance in primary infection but are necessary effectors in immunity to challenge infections (Lillehoj and Trout, 1994; Trout and Lillehoj, 1996). The importance of $CD8^+$ T cells in protective immunity to *E. tenella* infections is also suggested by the observation of a sharp transitory increase in the number of these cells in the peripheral blood (Breed *et al*., 1997) during primary infection. In fact, $CD8^+$ T cells seem to play dual role in the immune response to coccidial infections in chickens, as determined by two-colour immunofluorescence staining of infected gut from chicken strains differing genetically in susceptibility to coccidiosis (Lillehoj and Trout, 1994, Trout and Lillehoj, 1996). First, during primary infections with *E. acervulina* and *E. tenella*, the number of T cells coexpressing TCRγδ and CD8 markers increases in the epithelium. There they appear to be invaded by sporozoites. It is thought that these are the cells that transport sporozoites (Lawn and Rose, 1982; Al-Attar and Fernando, 1987; Trout and Lillehoj, 1993; Trout and Lillehoj, 1995) through the lamina propria to the crypt epithelium, where they exit and invade the crypt cells. Populations of $CD8^+$ cells also increase in the mucosa following challenge infection, where they frequently can be seen in close contact with infected cells. It is theorized that they are

functioning as cytotoxic cells, helping to eliminate the parasite challenge by killing the infected cells (Lillehoj and Trout, 1994).

Coccidia invade other leukocyte types in the gut mucosa. Sporozoites of *E. tenella* have been found in heterophils (Rose and Lee, 1977) and merozoites of *E. tenella* have been found in goblet cells and mast cells (Daszak *et al*., 1993). The relevance of these observations to immune control of avian coccidiosis is not yet clear. Populations of cells bearing markers (low-level $CD8^+$ and asialo-$GM_1$) for natural killer (NK) cells increase early during primary infections (Cheung and Lillehoj, 1991; Lillehoj, 1989). These cells exhibit NK activity in vitro (Chai and Lillehoj, 1988) and thus may be involved in immune surveillance. Mucosal macrophages phagocytize sporozoites during primary and challenge infections, but this activity does not seem enhanced in the immune chicken (Trout and Lillehoj, 1995).

Indeed, the ability of hens to transfer remarkable quantities of antibodies to their hatchlings through the egg yolk has long been appreciated, including in regard to the transfer of antibodies that protect chicks from infection with *E. tenella* (Rose and Long, 1971) or *E. maxima* (Rose, 1972). In many of the progeny from hens deliberately infected with high doses of *E. maxima*, this maternal immunity can be absolute, at least during the first week post-hatching (Smith *et al*., 1994a). Smith *et al*. (1994b) reported that, maternal immunity induced by *E. maxima* confers partial protection against *E. tenella*, possibly via cross-recognition of conserved proteins (or, at least, epitopes) in different *Eimeria* species. This report was later supported by the findings of Wallach *et al*. (1995; 2008) which shows that maternal immunization with conserved macrogametocyte proteins protect hatchlings against multiple species of *Eimeria* (Wallach *et al*., 1995;

2008). Additionally, it was demonstrated more recently (Lee *et al*., 2009a; b) that *Eimeria* specific antibodies purified from egg yolks of immunized hens can be used to transfer passive immunity against several species of poultry coccidia; the protection can be achieved via injection or oral delivery of the antibodies. Maternal immunization, therefore, appears to be a phenomenon that can be harnessed to control poultry coccidiosis.

## 2.9. Prevention and Control of Coccidiosis

### 2.9.1. Chemotherapy

The modern poultry industry may never have developed to its present extent without the advent of drugs to control coccidiosis. Today, the prevention and control of coccidiosis continue to rely hugely on chemotherapy and chemoprophylaxis using anticoccidial drugs (Chapman, 2001; 2008).

Anticoccidial drugs fall into two categories, the synthetic compounds popularly known as 'chemicals' that have specific modes of action against parasite metabolism, such as amprolium, clopidol decoquinate, halofuginone ; and the ionophore antibiotics, such as monensin lasalocid, salinomycin, narasin, and maduramycin, which act through general mechanisms of altering ion transport and disrupting osmotic balance (Taylor *et al*., 2007). The synthetic drugs were the first to be discovered and comprise a diverse array of molecules that are absorbed into the blood stream of the host and kill developing parasites in epithelial cells of villi in the intestine. One of the oldest of the synthetic drugs, nicarbazin, is also one of the most successful and is still used widely today (Chapman, 1994a). The ionophores usually targets and destroy the motile stages of the *Eimeria* life cycle (sporozoites and merozoites) in the gut lumen or following cell penetration (Smith

and Strout, 1979). Since the introduction of Monensin in 1972, ionophores have been the most widely used anticoccidial drugs for the control of coccidiosis (Chapman, 2001).

Most anticoccidial products frequently have more than one biochemical effect upon a specific developmental stage of Coccidia. Broad categorization of the mode of action of anticoccidials on the parasite metabolism has been undertaken (Chapman, 1997). Several of these anticoccidial products influence essential biochemical pathways of the parasitic cell by affecting an important co-factor of named pathway (Greif *et al.*, 2001). For example Ethopabate, which is often used in combination with Amprolium to improve the spectrum of efficacy, is a folate antagonist and blocks a step in the synthesis of para amino benzoic acid (PABA) and prevents the formation of nucleic acids and the synthesis of vitamins (Rogers *et al*., 1964). Ethopabate affects the second generation of schizonts (Reid, 1975) and is most active against *E. maxima* and *E. brunetti*.

Sulphonamides were the first drugs to be extensively used for the control of coccidiosis and also inhibit the folic acid pathway (Reid, 1975). Similar to ethopabate, the synthesis of dyhydrofolate is prevented by interfering with the dihydropteroate synthetase reaction, blocking the conjugation of pteridine and PABA (Reid, 1975). Thiamine analogues, like amprolium block the absorption of thiamine completely and have probably an antagonistic effect on the vitamin B1 supply (James, 1980).

Quinolone drugs such as buquinolate, decoquinate and nequinate, show anticoccidial activity at very low concentrations (Ryley and Betts, 1973). These products inhibit the respiration of coccidia by blocking the electron transport in their mitochondria (Wang, 1975). Although they are relatively safe and do not influence the egg production, egg

quality and feed conversion, resistance is rapidly induced (McManus *et al*., 1968). This has significantly limited their use in the field. Quinolones are found to be effective against *E. acervulina*, *E. brunetti*, *E. maxima* and *E. mivati* and to a lesser degree to *E. necatrix* and *E. tenella* (Ryley and Betts, 1973).

The true mode of action of nicarbazin (4, 4'-dinitrocarbanilide) is unknown. However, the product has been shown to inhibit both, the succinate-linked NAD reduction in mitochondria of bovine hearts and the energy dependent transhydrogenase and accumulation of $Ca^{2+}$ ions by rat liver mitochondria (Dougherty, 1974). It is not suitable for layers as it affects egg production and egg quality negatively. It may cause yolk mottling and eggshell depigmentation. Nicarbazin mainly affects the second generation of schizonts and to a lesser extent the other parasite stages (McLoughlin and Wehr, 1960). It is most effective against *E. tenella*, *E. necatrix* and *E. acervulina* and less to *E. maxima* and *E. brunetti*. Development of immunity is not inhibited.

Another anticoccidial drug possibly affecting mitochondrial function is the triazinetrione compound toltrazuril, which is applied in drinking water for preventive and therapeutic treatment (Harder and Haberkorn, 1989). Toltrazuril is efficacious against all intracellular stages (schizogony and gametogony) of all important *Eimeria* spp. in the chicken (Mehlhorn *et al*., 1988). It induces cidal changes in the organelles of the parasite at multiple levels, and it does not seem to impair the development of natural immunity (Greif, 2000).

Polyether antibiotics, commonly known as ionophores, with anticoccidial activity influence the transport of mono- or divalent cations (Na+, K+ and Ca++) across cell

membranes inducing osmotic damage (Shumard and Callender, 1967). These drugs are accumulated by extracellular stages (like sporozoites and merozoites) of the parasite in the lumen of the intestine (Long and Jeffers, 1982; Mehlhorn *et al*., 1983).

Monensin has a good efficacy against all *Eimeria* spp. although the efficacy seems to be less against *E. tenella* (Folz *et al*., 1988). Semduramycin showed a good efficacy against all *Eimeria* spp. and an excellent protection against *E. maxima* (Logan *et al*., 1993; Conway *et al*., 1995). Narasin has a good efficacy against all *Eimeria* spp. more or less comparatively to monensin (Ruff *et al*., 1979; Ruff *et al*., 1980; Walters *et al*., 1981). Salinomycin has a broad anticoccidial spectrum against all *Eimeria* spp. comparable to or more effective than monensin and lasalocid (Bedrnik, *et al*., 1980; Greuel and Raether, 1980; McDougald *et al*., 1981). In general, treatment with ionophore anticoccidials allows some level of infection and consequently the development of immunity against coccidia, however this depends upon the dosage used, the *Eimeria* spp. and the infection level (Chapman and Hacker, 1993; Chapman, 1999b).

Diclazuril shows a coccidicidal effect towards all *Eimeria* spp. of the chicken; however its exact mode of action has not been unraveled yet. It is a nucleoside analogue thought to be involved in acid nucleic synthesis, possibly affecting later phases of coccidia differentiation (Verheyen *et al*., 1988). It has been shown to affect parasite wall synthesis resulting in the formation of an abnormally thickened, incomplete oocyst wall and zygote necrosis in both *E. brunetti* and *E. maxima* (Verheyen *et al*., 1989). Diclazuril has recently been shown to induce ultrastructural changes in merozoites and cause disruption of transmembrane potential in the mitochondrion (Zhou *et al*., 2010).

It is quite clear, however, that some degree of resistance to all anticoccidial drugs, has developed (Chapman, 1994; Chapman, 1998; Abbas *et al*., 2011). To minimize the effects of resistance, poultry producers rotate the use of various anticoccidials with successive flocks, combine chemical and ionophore treatments, or employ shuttle programs during a flock grow out. Application of these treatment programs depends on seasonal conditions and prevalence of various species of coccidia (Williams, 1998). However, the success of most anticoccidial drugs used in the field is basically because they only partially suppress parasite development, allowing birds to acquire natural immunity as a consequence of exposure to parasites that escape drug action (Chapman, 1999).

### 2.9.2 Vaccination

The major alternative to chemotherapeutic control of *Eimeria* is vaccination with live vaccines and it depends upon immune protection induced by vaccination with oocysts containing different formulations of live wild-type or attenuated parasites of one or more species (Shirley *et al*., 1995; Shirley *et al*., 1997). After ingestion, the oocysts liberate sporozoites into the intestine in which multiple endogenous rounds of asexual replication precede a single phase of sexual replication, which provides an opportunity for cross-fertilisation and genetic recombination (Rollinson *et al*., 1979; Sutton *et al*., 1986; Shirley and Harvey, 2000). Host immune responses to *Emiria* infection are complex, strongly influenced by host genotype, infection history and parasite dose (Lillehoj, 1988; Bumstead and Millard, 1992; Smith *et al*., 2002; Blake *et al*., 2004; Blake *et al*., 2005).

All seven *Eimeria* species that infect chicken are immunogenic, most notably *E. maxima*, for which infection with a single sporocyst is capable of stimulating a protective immune

response (Long *et al*., 1976; Lee and Fernando, 1978). Infection with as few as five *E. maxima* oocysts can induce complete protective immunity (Blake *et al*., 2005). Protective immunity has been shown to be both parasite species-specific and, in some cases, strain-specific (Smith *et al*., 2002), but the genetic basis to this phenotype remains unknown. For example, infection of Line C chickens with either the Houghton (H) or Weybridge (W) strains of *E. maxima* confers complete immune protection against subsequent challenge by the homologous strain (no detectable oocyst output), but no apparent protection against challenge by the heterologous strain (Smith *et al*., 2002). Whilst *Eimeria* species may be considered to be antigenically complex throughout their life-cycle (Abrahamsen *et al*., 1993; Wan *et al*., 1999; Tabares *et al*., 2004), it is the early endogenous developmental stages that are of most importance to the induction of a protective immune response (Jeffer and Long, 1985; Jekins *et al*., 1991). Nonetheless, other stages of the life-cycle, including the sexual stages, have also been shown to be inhibited by protective immunity (Rose and Hesketh, 1976).

*2.9.2.1 Vaccination with wild-type Eimeria species*

While all *Eimeria* spp. are immunogenic, there is a hierarchy with *E. maxima*, *E. brunetti* and *E. praecox* regarded as the most immunogenic species (Rose, 1976; 1987) and *E. tenella* and *E. necatrix* as the least. This variation is a factor in the composition of live vaccines and the numbers of oocysts of each species that are included may vary considerably.

The first vaccine to target an *Eimeria* parasite (DM® Caecal Coccidiosis Vaccine; Dorn and Mitchell Inc., USA) was introduced in 1952 and contained live, wild-type, oocysts of only *E. tenella* (Williams, 2002). Three further species were later added and the

vaccine was sold as Coxine® in the United States and CocciVac® in Europe (Schering-Plough Animal Health (Williams, 2002). The ability of live wild-type parasite vaccines to induce potent protective responses has ensured that CocciVac® and later products, such as Immucox® (Vetech Laboratories, Canada, marketed from 1985 (Williams, 2002), remain commercially successful. About 2500 million doses of these vaccines were sold between 2001 and 2004 (Shirley *et al*., 2005) and some new products, based on live oocysts of different strains and numbers, such as Inmuner® Gel-Coc (Vacunas Inmuner, Argentina), have been introduced more recently.

A key consideration in the application of virulent wild type vaccines is that optimal vaccination is achieved by an even, low-level exposure across the vaccinated group (Shirley *et al*., 2005) because any variable exposure significantly elevates the risk of impaired performance and disease. Thus the delivery of vaccines has been investigated extensively and dosing strategies now include the addition of oocysts to feed or water, preparation as gel-blocks and direct application *via* sprays to the hatched birds or injections to the embryonating egg (Chapman *et al*., 2002).

*2.9.2.2 Vaccination with attenuated Eimeria species*

The use of attenuated parasites offers a significantly safer approach to vaccination and attenuation through the selection of 'precocious' parasites that are characterised by a reduced reproductive capacity (Jeffers, 1975) is now the preferred option. The parasites thus obtained are characterised by an abbreviated generation time due to the reduction, or complete loss, of one or more rounds of asexual multiplication (McDonald and Shirley, 1984; McDonald *et al*., 1986a; b). Whilst the attenuation process has been shown to reduce oocyst output between 72 and 97% compared with the non-attenuated parent,

minimising the risk of clinical disease (Shirley *et al*., 2005); immunogenicity comparable with the wild-type parent is retained (Shirley and Millard, 1986; McDonald *et al*., 1986a; b). Attenuation by serial passage of parasites in the chorioallantoic membranes of embryonating eggs has been used for *E. tenella* and an egg-adapted line has been included in the Livacox® vaccines (Biopharm, Czech Republic (Shirley and Bedrnik, 1997; Shirley *et al*., 1997).

The requirements for control of coccidiosis in the breeding and commercial egg layers, and broiler sectors of the poultry industry are different and reflect the value of individual birds and the length of time they are reared (Blake and Tomley, 2014). Live attenuated vaccines were rapidly introduced into the former sector and include Paracox® (Schering-Plough Animal Health, UK) and developed with the IAH (McDonald and Shirley, 1984; McDonald *et al*., 1986a; Shirley and Millard, 1986; Shirley *et al*., 1995; Shirley *et al*., 1997) now registered for use in more than 30 countries and Livacox® (Biopharm, Czech Republic), registered in more than 20 countries (Williams, 2002). Both vaccines include formulations tailored to suit each sector of the poultry industry, formulations of eight and four species/strains to suit the breeder/layer sector and five and three species/strains to suit the broiler sector, respectively. The inclusion of two *E. maxima* strains in the Paracox® series addresses the characteristics of antigenic diversity of this species (Smith *et al*., 2002). The issue of antigenic diversity has provided scope for the development of regional alternatives (e.g. Eimeriavax® 4m, Bioproperties, Australia; Eimerivac® plus, Guangdong Academy of Agricultural Sciences, China).

Whilst vaccines comprising as many as eight lines of live oocysts are highly successful (Shirley *et al*., 1995; Shirley *et al*., 1997; Williams *et al*., 1999; Williams, 2002;

Chapman *et al*., 2002) and some offer further novel industrial-scale methods of administration, such as *in ovo* approaches (Inovocox®, Embrex, USA) (Weber and Evans, 2003; Weber *et al*., 2004), the costs of their production are high and shelf lives in aqueous media are limited by an inevitable sharp fall in viability after several months of storage. Hence, effective and more economically viable vaccines for controlling the *Eimeria* species are still required.

### 2.9.3 Recent advances with relevance to sub-unit vaccine development

The *Eimeria* parasite has multiple stages of life cycle, some of which require host cells while others are host independent. In order to invade the host cells, the parasite must attach to the gut epithelial cells. The invasion of host cells by *Eimeria* spp. is a complex, multistep process that begins with the apical attachment of the parasite to the host cell (Tabares *et al*., 2004). This is followed by rapid internalization to form an intracellular, parasitophorous vacuole (PV) in which the newly invaded parasite becomes enclosed, enabling its survival within the host (Tabares *et al*., 2004). During the invasion process, specialized secretory organelles known as micronemes, rhoptries and dense granules deliver cargo proteins in a co-ordinated fashion. These secreted proteins are implicated in playing a central role in invasion and the establishment of infection by *Eimeria* parasites (Daszak, 1999; Tomley and Soldati, 2001; Soldati *et al*., 2001; Carruthers, 2002; Sasai *et al*., 2008). The other molecules implicated for the host cell invasion by *Eimeria* parasites are the glycosylphosphatidylinositol (GPI) - anchored proteins that are collectively referred to as surface antigens or SAGs that coat the surfaces of the invasive stages of *Eimeria* parasites (Gurnett *et al*., 1990; Tabare´s *et al*., 2004). Thus, to develop a prophylactic drug or vaccine against coccidiosis, it appears logical to target this attachment and internalisation step of *Eimeria* into the host cell. In this regard

considerable efforts are ongoing to characterise *Eimeria* parasite molecules that mediate early interactions with the host.

To date a small number of parasite antigens have been studied in terms of their ability to induce 'protective' immune responses and these represent a tiny proportion of the potential antigens encoded by the parasite genome (Shirley *et al*., 2004).The availability of a full genome sequence in the *Eimeria* genome consortium has dramatically increased the knowledge of the parasite and has led to projects that utilize high-throughput global approaches for *Eimeria* protein identification (Bromley *et al*., 2003; deVenevelles *et al*., 2004).

The use of subcellular fractionation to isolate microneme and rhoptry organelles (Kawazoe *et al*., 1992; Tomley, 1997) combined with molecular cloning and sequencing has made a major contribution to understanding the role of *Eimeria* proteins during invasion of the epithelial lining of the gut mucosa of the host (Tomley *et al*., 1991; 1996; 2001; Brown *et al*., 2000; 2001; 2003; Labbe *et al*., 2005; Periz *et al*., 2005).

Micronemes are the smallest of the secretory organelles and they contain complexes of transmembrane and soluble protein called the microneme proteins (MICs) that are discharged from the apical tip early in invasion (Carruthers and Sibley, 1999; Bumstead and Tomley, 2000; Rabenau *et al*., 2001).These proteins are members of TRAP (thrombospondin-related anonymous protein) family of proteins containing one integrin-insertion-I domain, five thrombospondin type I modules (TSP I) and conserved transmembrane and C-terminal regions (Tomeley *et al*., 1991; Tomley and Soldati, 2001) and during invasion they bind the parasite surface, becoming concentrated at the point of invasion (Tomley *et al*., 1996) and eventually released from the posterior end. In *E.*

*tenella*, five MIC proteins have been described in detail so far and many more, together with rhoptry and dense-granule proteins are emerging from proteomics studies (Bromley *et al*., 2003).

Microneme proteins are essential for parasite motility and compounds that interfere with their secretion are extremely effective at blocking parasite attachment and invasion in vitro and parasite infectivity *in vivo* making them attractive targets for therapy against coccidiosis (Wiersma *et al*., 2004). In *E. tenella*, five MIC proteins have been described so far and many more, together with rhoptry and dense-granule proteins are emerging from proteomics studies (Bromley *et al*., 2003). Preliminary studies by Subramanian *et al.* (2008) indicated that immunization of birds with recombinant microneme-1 protein of *Eimeria tenella* EtMIC1 is able to impart partial protection against homologous challenge in chicken.

Apical membrane antigen-1 (AMA-1) is another micronemal protein of apicomplexan parasites that appears to be essential during the invasion of host cells by the invasive stages of the apicomplexan parasites (Alexander *et al.*, 2005; Besteiro *et al*., 2009; Santos *et al*., 2011; Lamarque *et al*., 2011; Cortidor *et al*., 2011; Jiang *et al*., 2012). It was first identified as a conserved antigenic protein in the malaria parasite *Plasmodium knowlesi* (Thomas *et al*., 1984).Together with rhoptry neck proteins (RONs), AMA-1 is involved in the formation of the moving junction complex, which is a circumferential zone that moves backward and eventually pinches the PV from the host cell membrane (Alexander *et al*., 2005; Besteiro *et al*., 2009; Curtidor *et al*., 2011; Lamarque *et al*., 2011; Santos *et al*., 2011).

*Plasmodium falciparum* AMA-1 (PfAMA-1) has been demonstrated to induce protective immunity against the parasite challenge in animal models (Remarque *et al*., 2008). Similarly, preliminary reports have established that recombinant protein vaccine derived from AMA-1 of *E. maxima* and *E. tenella* has conferred significant immune protection against experimental challenge by the respective parasites (Blake *et al*., 2011a; Jiang *et al*., 2012). Apical membrane antigen-1 has also been identified as an immunoprotective protein from other apicomplexan parasites, such as *Babesia*, *Toxoplasma*, *Neospora* and *Theileria* (Donahue *et al*., 2000; Gaffar, 2004; Zhang *et al*., 2007; Tonukari, 2010). Interestingly, the homologues of the AMA-1 antigen appears to be protective against a range of apicomplexan parasites including *Plasmodium* spp., which suggest that there may be some protective antigens shared across this diverse group of parasites (Blake *et al*., 2011a). According to Jiang *et al*. (2012) *Eimeria tenella* AMA-1 has an open reading frame of 1608 bp encoding a protein of 535 amino acids and is express at higher level in sporozoites than in unsporulated oocysts, sporulated oocysts and second generation merozoites.

Along with the discovery of the gene encoding *E. maxima* AMA-1 using a population-based genetic mapping approach allied with a protection based screen of candidate genes, Blake *et al*. (2011a) reported another protective antigen termed 'immune mapped protein-1' (IMP-1) in *E. maxima*. Homologues of the IMP-1 antigen have also been identified in *Toxoplasma gondii* and *Neospora caninum* (Blake *et al*., 2011a). IMP-1 has been demonstrated to be immunogenic and confer protection against *E. maxima* challenge in chickens (Blake *et al*., 2011a) and other apicomplexan parasites, such as *Toxoplasma gondii* and *Neospora caninum* (Cui *et al*., 2012a; b). Recently Yin *et al*. (2013) characterized and evaluated the immunogenicity of recombinant IMP-1 of *E. tenella*

(EtIMP-1) and in combination with molecular adjuvant (a truncated flagellin, FliC) which they discovered that immunization of chickens with the recombinant EtIMP1-truncated FliC fusion protein resulted in stronger cellular immune responses than immunization with only recombinant EtIMP1 with adjuvant.

The identification and characterization of AMA-1 and IMP-1 represent a substantial step towards development of an effective anticoccidial sub-unit vaccine and raises the possibility of identifying novel protective antigens for other apicomplexan parasites (Blake *et al*., 2011a; Jiang *et al*., 2012).

Rhoptries are club-shaped organelles connected by their 'neck' ends to the apical pole of the parasite. The proteins that reside within these organelles are classified in the coccidian parasite, *Toxoplasma gondii*, according to their localisation to either the anterior neck region (rhoptry neck proteins, RONs) or the posterior club/ bulb compartment (rhoptry proteins, ROPs) of the organelle (Bradley *et al*., 2005; Proellocks *et al*., 2010; Lamarque *et al*., 2012). Furthermore the two classes of proteins are differentially deployed during invasion with several RONs having a critical early role in the formation and function of the moving junction (Alexander *et al*., 2005; Lebrun *et al*., 2005), whereas the ROPs are secreted later into the newly formed parasitophorous vacuole or into the host cell (Carruthers and Sibley, 1997). *E. tenella* sporozoites's rhoptries contain at least 60 different proteins (Tomley, 1994) which were recently characterized by Oakes *et al.* (2013).

Alexander *et al*. (2005) and Lebrun *et al*. (2005) demonstrated that a complex of secreted RON proteins interacts with the microneme protein, AMA-1, and localises to the moving

junction of *T. gondii* tachyzoites during cell invasion. A phenomenon that was also confirmed to occur during cell invasion by erythrocytic stages of *Plasmodium* (Alexander *et al*., 2006; Cao *et al*., 2009; Collins *et al*., 2009).

Until recently, little was known about GPI-linked SAGs in *Eimeria*, but examination of ESTs from sporozoites and second-generation merozoites revealed two families of transcripts encoding potential GPI-linked variant surface proteins (Tabares *et al*., 2004). Using bioinformatics and wet-lab work 23 genes were shown to be differentially expressed during the parasite life cycle and confirmed to be surface-localised and GPI-anchored (Tabares *et al*., 2004).

Transcriptome, proteome and genome data indicate that *E. tenella* expresses up to 80 different SAG proteins, and confirms that these are differentially regulated during the life cycle such that second-generation merozoites are coated with more complex mixtures of SAGs than either sporozoites or first-generation merozoites (Lal *et al*., 2009; Novaes *et al*., 2012). Their surface location suggests that SAGs may induce potent immune responses that can, for example, block sporozoite invasion of cultured cells (Brothers *et al*., 1988). The co-expression by merozoites of highly polymorphic SAGs could render anti-SAG immune responses ineffective against these stages.

A study of 10 *E. tenella* merozoite-expressed SAGs showed that three of these induced an increase in nitric oxide production, IL-1b and IL-10 transcription, and induced a decrease in IL-12 and interferon-γ (IFN-γ) transcription in chicken macrophages (Chow *et al*., 2011). This indicates that at least a subset of SAGs has the ability to modulate chicken innate and adaptive immune responses, which may suppress cell-mediated

immunity and also contribute to the marked pro-inflammatory responses and associated pathology seen during *E. tenella* infection.

### 2.9.4 Management practice and biosecurity.

In most tropical countries like Nigeria, naturally ventilated poultry houses with controllable windows at the sides of the house is used (Adene and Oluleye, 2004). Proper ventilation brings in fresh air and takes out stale air. If the ventilation system is not effective, control of the poultry house with respect to temperature and relative humidity fails which will give an ideal condition for sporulation of the oocyst and this may lead to disease outbreak (Methusela, 2001; 2002). Although it is difficult to control coccidiosis by biosecurity measures alone, it is still an important component of good management practice which will control coccidiosis and other diseases with their associated economic losses (Methusela, 2001; 2002). Biosecurity practice includes hygiene, disinfection and litter management. The feeding and watering system should be placed in a way that chicken can easily reach and adequate spaces are available for feeding and watering (Blake and Tomley, 2014). Otherwise, contamination of feed and water with coccidia oocysts from chicken dropping is inevitable and hence increases the risk of infection and disease (Whiteman and Bickford, 1989). The birds should not be overcrowded. Prevention of damp areas in the house should be the practical emphasis of poultry producers (Taylor *et al*., 2007). Leaking roofs or watering troughs should be adjusted; redistribute the litters frequently to avoid concentration of the oocysts at places such as feeding and watering troughs. Litter should be kept dry to 25- 30% moisture (Soulsby, 1982). The house should be cleaned thoroughly before each new flock comes in. The walls and dirty floors should be washed out and disinfected (Whiteman and Bickford, 1989).

The coccidial infection should be kept on the outside. Disinfect shoes before entering the chicken house. Keep wild birds and other animals out, separate older birds from younger birds (Zander, 1978).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Sample Sites:

Litter samples were collected from 123 commercial poultry farms in Zaria, Sabon Gari, Igabi, Giwa, Makarfi, Ikara and Soba Local Government areas of Kaduna State, Nigeria from December, 2012 to April 2013 (Figure 1).

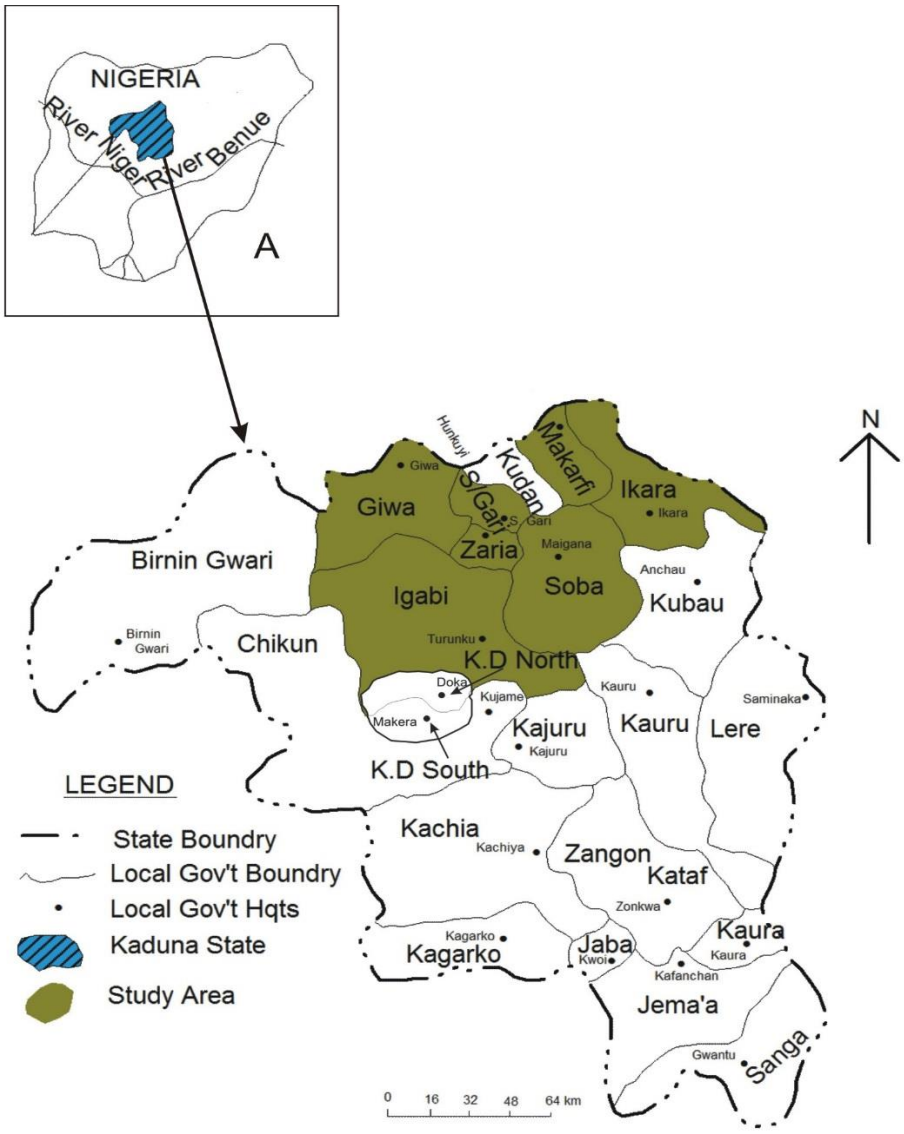



Figure 1: Map of Nigeria (A) showing Kaduna state and the study area.

Source: Department of Geology, Ahmadu Bello University, Zaria.

Sample size: The sample size was determined using the formula outlined by Thrusfield, (1997) as follows:

$$n = Z^2pq/d^2$$

Where: n = sample size

z = value for standard normal deviate = 1.96

p = anticipated prevalence

q = 1-p

d = desired precision = 0.05

Using a prevalence rate of 14 % reported by Adamu *et al.* (2009) the calculated sample size was computed as follows:

$$n = \frac{(1.96)^2 \times 0.14 \times 0.86}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.1204}{0.0025} = \frac{0.46253}{0.0025} = 185$$

The poultry farms were selected and sampled based on availability and accessibility to the farms and the willingness of the poultry farmers to participate in the study. Flock record such as bird type, age, flock size, management system (Deep litter or battery cage) and anticoccidial medication used were recorded from each farm visited. Physical health status of the sampled flocks were also assessed by observing the flock activity and alertness before and during sampling.

Due to logistic reasons (many of the large and medium scales farms visited were not willing to participate in the study) only 123 flocks were sampled as against the calculated sample size of 185.

### 3.2 Collection of Litter/Faecal Samples

Litter/faecal materials were collected in each poultry house at different spots following a roughly W-shaped path starting and finishing in the corners of one of the long sides of the house. Along this path, portions of the litter/faecal materials were manually but aseptically collected and placed in clean plastic bags and transported on ice pack to the Research Laboratory of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria for processing.

### 3.3 Laboratory Processing of the Samples.

In the laboratory, 200g of each sample was weighed, transferred into a plastic beaker, homogenized using glass rod and soaked in about 500 ml of distilled water overnight. The soaked samples were then homogenized by thorough stirring using a glass rod and were subsequently filtered through a metal sieve. The filtrate from each sample was allowed to sediment for about an hour on the laboratory bench after which the supernatant fluid was discarded into a clean beaker. About 10 ml of the sediment was then transferred into a centrifuge tube and tested for the presence of *Eimeria* oocysts using the simple floatation technique as described by Soulsby, (1982).

For the samples positive for *Eimeria* oocysts, the number of oocysts were estimated as described by Lawal *et al*. (2008) as follows: 1-10 oocysts per field = +1, 11-20 oocysts per field = +2, >20 oocysts per field = +3. For the purpose of this study only samples

with oocysts counts of +2 and +3 were selected for further processing and on that basis a total of 14 samples representing 14 commercial poultry farms in the study area were selected for further processing and subsequent study.

### 3.4 Harvest and Sporulation of Oocysts from Selected Samples

Oocysts were harvested from the sedimented positive samples by the centrifugal floatation technique (Soulsby, 1982) as follows: Each sediment was transferred into centrifuge tubes, mixed with twice volume of saturated salt solution and centrifuged at 300 × *g* for 10 minutes. Coccidia oocysts were harvested from the surface of the supernatant by pipetting about 2 ml from the surface into a test-tube, diluted 10 folds with distilled water and centrifuged at 300 × *g* for 10 min to sediment the oocysts to the bottom of the test-tube and the water decanted. The harvested oocysts were resuspended in distilled water and washed by centrifugation three to four times to remove the flotation solution. The sediment containing the oocysts was transferred into Petri-dishes mixed with 2.5% (w/v) potassium dichromate solution and allowed to sporulate at room temperature for 7 days. After sporulation the potassium dichromate solution was washed off by centrifugation at 300 × *g* for 5 min, three times with distilled water. The washed sporulated oocysts were then transferred into a clean 20 ml screw cap tubes; fresh potassium dichromate solution was added to the tubes, labelled appropriately (as Nig1 to Nig14 respectively) and stored at $4^0$C until needed.

### 3.5 Propagation of Oocyst in *Eimeria* Free Broiler Chicks

Seventy (70) day-old Cobb broiler chicks where obtained from a commercial hatchery and brooded for two weeks under strict biosecurity measures in cages. During the brooding period the faeces of the chicks were examined for the presence of coccidia

oocysts every 3 days and were found to be negative in all cases. At two weeks of age the birds were grouped into 14 groups of 5 birds each and housed in separate well cleaned and fumigated (with 20% formalin) cages. Birds from cage 1 to 14 were individually inoculated orally with approximately 5000 sporulated oocysts of isolate Nig1 to Nig14 respectively in order to amplify sufficient oocysts for the subsequent studies. The birds were monitored under strict biosecurity with feed and water provided *ad libitum*.

In order to enrich the samples with *E. tenella* (which is the main target species for the subsequent studies) the infected birds from each group were humanely sacrificed at day 7 post infection and the caeca were collected at post-mortem for caecal harvest of Coccidia oocysts.

### 3.6 Caecal Harvest and Sporulation of the Propagated Oocysts

Coccidia oocysts were recovered from the harvested caeca as described by Shirley, (1995) as follows: The harvested caeca were carefully cut open longitudinally and placed on a glass plate to minimise the likelihood of losing any material. By holding the edge of each caecum a glass microscope slide was used to carefully scrape the caecal contents, plus the mucosal and the deeper muscle layers, away from the remaining tissue and placed into a beaker containing phosphate buffered saline (PBS). The scrapings were homogenized using a glass rod in the beaker. 1.5% (w/v) trypsin was added to the homogenate (to break down any connective tissue) and incubated in a water bath at 41$^{o}$C for between 30 to 90 minutes. The beaker was swirled occasionally during the course of incubation. The trypsin treated homogenate was strained through a double layer of muslin into a centrifuge tubes and centrifuged at ~750 × *g* for 12 minutes. The supernatant was poured off and replaced with distilled water and centrifuged at ~750 × *g* for 12 minutes.

The washing was repeated three times using distilled water. The sediment containing the oocysts was transferred into Petri-dishes, mixed with 2.5% (w/v) potassium dichromate solution and allowed to sporulate at room temperature for 7 days. After sporulation the potassium dichromate solution was washed off by centrifugation at ~750 × *g* for 12 min three times with distilled water. The washed sporulated oocysts were then transferred into a clean 20 ml screw capped tubes; fresh potassium dichromate solution was added to the tubes, labelled appropriately (as Nig1 to Nig14) and stored at $4^0$C.

### 3.7 Shipping of the *Eimeria* Oocysts to the United Kingdom.

The screw topped plastic tubes containing 20 ml *Eimeria* sporulated oocysts suspension in 2.5% (w/v) potassium dichromate solution of the selected isolates were sealed with parafilm and double-bagged in sealed plastic bags. The packaged was then shipped at ambient temperature to the Parasite Laboratory, Department of Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, United Kingdom under the IAPO permit PATH/71/2011/14.

### 3.8 Washing and Counting of the Sporulated *Eimeria* Oocysts Samples

On arrival to the parasite laboratory of the Royal Veterinary College the *Eimeria* oocyst samples were washed from the tissue and faecal debris as described by Shirley, (1995) as follows:

The tubes were transferred to a centrifuge and centrifuged at ~ 750 × *g* for 10 minutes at room temperature to pellet the oocysts. The supernatant was carefully poured off into a beaker, and checked microscopically (x10/x20) for any oocysts that may have failed to pellet, prior to discarding. Distilled water was added to the oocyst pellet, mixed and centrifuged at ~ 750 × *g* for 10 minutes. The washing was repeated at least twice, until

all trace of the orange potassium dichromate has been removed. The oocyst pellet was then suspended in 10% sodium hypochlorite solution for 5 mins and swirled intermittently. After sodium hypochlorite treatment, the preparation was topped up with distilled water, mixed and centrifuged at ~ 750 × *g* for 10 minutes to pellet the oocysts. The supernatant was discarded after microscopic examination. The pellet, (brown to snowy white in colour) was re-suspended in saturated salt solution (Sodium chloride) and mixed with a Pasteur pipette to ensure that the pellet was thoroughly re-suspended, leaving no lumps. The tube was filled to within 2 cm of the top and overlaid with distilled water by gently running the water down the side of the tube with a Pastette and centrifuged at ~ 750 × *g* for 10 minutes. After the centrifugation the oocysts were collected at the interface between the salt and water phases with a sterile, disposable plastic Pastette and dispensed into a new tube with an equal quantity of water. The collected oocysts were then centrifuged at ~ 750 × *g* for 10 minutes. The supernatant was discarded after removal from the pelleted oocysts and microscopic examination; the tube was topped up with distilled water as a wash and centrifuged at ~ 750 × *g* for 10 minutes. The washing step was repeated three times. After the final wash the cleaned oocysts were re-suspended in a suitable volume of distilled water and enumerated using a modified-Fuchs Rosenthal counting chamber. The tubes containing the cleaned oocysts were clearly labelled with isolate number, date and amount of oocysts/ml; and stored at 4$^{o}$C until required.

### 3.9 Re-propagation of the Washed Oocyst in Pathogen Free Light Sussex Chicks

Due to the low oocyst counts and poor sporulation rates observed from the washed oocyst samples, 9 sample (Nig1-5, Nig7 and 8; Ngi11 and 12) out of the 14 samples were re-passaged in three week old pathogen free Light Sussex chickens as follows:

The birds were housed in pathogen free wire cages in 9 groups of two birds per cage under strict biosecurity. Birds from each cage were inoculated with an isolate of *Eimeria* oocysts by oral gavage and at day 7 post inoculation the birds were humanely sacrificed by cervical dislocation and their caeca collected post-mortem for harvest of *Eimeria* oocysts. The harvested caeca were longitudinally cut open and examined for post-mortem lesions consistent with caecal coccidiosis. The lesions observed were scored based on the scoring method of Johnson and Reid (1970). Caecal contents were carefully scooped without scraping the underlying mucosal tissues and placed into 50 ml falcon tubes after which deep scrapings of the caecal mucosa were collected and placed into a separate tube. The tubes were separately treated with trypsin as described earlier, washed with distilled water and the sediment containing the oocysts were separately sporulated in 2.5% (w/v) potassium dichromate solution as follows:

For each tube of recovered oocysts approximately 25 ml of 2.5% (w/v) potassium dichromate solution was added and the mouth of the tube sealed with a parafilm. Five or six perforations were made at the sealed end to ensure aeration after which the tubes were arranged and supported vertically in a beaker and placed on a shaker set at low speed. The samples were shaken for 4 days at room temperature to allow for sporulation of the recovered oocysts. After sporulation, the oocysts were washed by sodium hypochlorite treatment, harvested by the centrifugal floatation technique, enumerated and stored as described above.

### 3.10 Morphometric Identification of *Eimeria* Species in the Processed Samples.

For morphometric identification of the *Eimeria* species composition in each sample, 5 µl of each oocyst suspension was placed on a glass slide, cover slipped and examined at

×100 objective lens of an Olympus CX 41 light microscope using oil immersion. About 30 -50 oocysts were then photographed at random using the Olympus CX 41 microscope coupled with Olympus DP20 camera. The lengths and widths of the photographed oocysts were subsequently measured with the assistance of the software ImageJ 1.45s (Wayne Rasband National Institutes of Health, USA), after which their shape index (length/width) were calculated for species identification as previously described by Long and Reid (1982) as follows: 1.09 = *Eimeria mitis*, 1.19 = *Eimeria necatrix*, 1.16 = *Eimeria tenella*, 1.24 = *Eimeria praecox*, 1.25 = *Eimeria acervulina*, 1.31 = *Eimeria brunetti* and 1.46 = *Eimeria maxima*.

### 3.11 Molecular Identification of *Eimeria* Species in the Processed Samples

#### 3.11.1 Disruption of oocysts walls by glass beads beating

A sub-sample of 4 ml of each washed oocyst suspension containing about 4 to 5 x $10^6$ sporulated oocysts were pelleted by centrifugation at ~750 × *g* for 10 min. About 3 ml of the supernatant was carefully pipetted and discarded while the oocyst pellets were re-suspended in the remaining 1 ml of the distilled water, transferred into 1.5ml screw top Eppendorf tubes and re-pelleted by centrifugation at ~750 × *g* for 10 min. The supernatant was discarded and the pellet was re-suspended in 500 µl sterile TE buffer after which an equal volume of sterile size 8 glass beads (~0.25-0.5 mm in diameter (Sigma Aldrich, USA) were added. The oocysts were then disrupted by bead beating using a Mini Beadbeater-8, (Biospec Products, Bartlesville, USA) for two minutes. The tubes containing the smashed oocysts, glass beads and TE buffer homogenate were immediately placed on ice before the commencement of the total genomic DNA extraction.

### 3.11.2 Total genomic DNA extraction using the QIAamp DNA tissue mini kit

Total genomic DNA was isolated from the smashed oocyst homogenate using a QIAamp DNA Tissue mini kit (Qiagen, Germany) as per the manufacturer's protocol. Briefly, 100 µl of buffer ATL was added to the homogenate, vortexed and pipetted into a new 1.5 ml Eppendorf tube, leaving only the glass beads in the previous tube. 20 µl of proteinase K was added, mixed by vortexing and incubated at $56^0$C for 10 min. 200 µl of buffer AL was added to the mixture, mixed by pulse-vortexing for 15s and was incubated at $70^0$C for 10 min, after which 200 µl of 100% ethanol was added and again mixed by pulse-vortexing for 15 s. The mixture was then poured into the QIAamp Mini spin column (placed in a 2 ml collecting tube) and centrifuged at 6000 × *g* for 1 min. The QIAamp Mini spin column was placed into a new clean 2ml collecting tube and the tube containing the filtrate was discarded. 500 µl buffer AW1 was added to the column and centrifuged at 6,000 × *g* for 1 min after which the collecting tube containing the filtrate was discarded and the spin column was placed into another clean 2 ml collection tube. The QIAamp spin column was carefully opened and 500 µl of buffer AW2 was added and centrifuged at full speed (20, 000 × *g*) for 3 min. The collection tube containing the filtrate was discarded; the column was placed into a new collection tube and centrifuged at full speed for 1 min to eliminate the chance of possible buffer AW2 carryover. The collection tube was then discarded and the QIAamp spin column was placed into a sterile 1.5 ml collection tube. The genomic DNA was eluted by adding 200 µl buffer AE to the column and incubated at room temperature for 1 min, after which it was centrifuged at 6,000 × *g* for 1 min. The QIAamp spin column was placed into a new 1.5 ml collection tube to repeat the elution step, while the old collection tube containing the eluted DNA was appropriately labelled and the DNA yield was measured at absorbance of 260 and 280 nm using a nanodrop to assess quality and quantity.

### 3.11.3 Identification of *Eimeria* spp. by nested PCR

A standardized nested PCR protocol using ITS-1 primers for identification of *Eimeria* species of poultry was used (Table 3.1). Primers amplifying the entire ITS-1 sequence with flanking partial 18S rDNA and 5.8S rDNA regions of *Eimeria* were used in the genus-specific PCR phase, while species-specific primers targeting the ITS-1 region were used to amplify the individual *Eimeria* species as described by Lew *et al*. (2003).

Each PCR reaction contained 2 µl genomic DNA template, 25 pmol forward and reverse primer, 0.5 U *Taq* polymerase (Invitrogen, Paisley, UK), 10mM Tris-HCl, 1.5mM MgCl2, 50mM KCl and 200 µM dNTPs. The thermal cycling condition included an initial denaturing step at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec and a final extension at 72°C for 7 min using Gstorm thermal cycler. 1.0 µl of the product of the primary PCR reaction was used as template in a 25.0 µl second round reaction mixture for the nested PCR with species-specific primers in individual tubes, using the same amplification conditions described above except for different annealing temperatures for different *Eimeria* spp. (58°C for *E. mitis*; 61°C for *E. necatrix* and *E.* praecox; 65°C for *E. tenella*; 71°C for *E. acervulina*, *E. maxima* and *E. brunetti)*. Positive controls containing known *Eimeria* species and Negative, no-template controls were included with each assay. The amplification products of the specific nested PCR were analysed by gel electrophoresis in 2% agarose gels in 1xTAE buffer stained with SafeView nucleic acid dye.

### 3.11.4 Identification of new operational taxonomic units (OTUs) of chicken *Eimeria* based on ITS-2 PCR.

A polymerase chain reaction protocol using internal transcribed spacer 2 (ITS-2) primers (Table 3.1) for identification of the OTUs of chicken *Eimeria* were used to survey the

samples for OTU-Y and OTU-Z sequences in the genomic DNA extracted from the oocysts sampled of this study. OTU-X was not surveyed surveyed using the ITS-2 PCR because there were no primers available at the time of the study.

The primers were designed based on the sequences of the ITS-2 of the OTUs of chicken *Eimeria* characterized and published by Fornace *et al*. (2013). The PCR was carried out in a 25.0 µl PCR reaction mixture which included 2 µl genomic DNA template, 25 pmol forward and reverse primer, 0.5 U *Taq* polymerase (Invitrogen, Paisley, UK), 10mM Tris-HCl, 1.5mM MgCl2, 50mM KCl and 200 µM dNTPs. The thermal cycling condition included an initial denaturing step at 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec and a final extension at 72°C for 10 min using Gstorm thermal cycler. The PCR products were analyzed by gel electrophoresis in 2% agarose gels in 1xTAE buffer stained with SafeView nucleic acid dye.

### 3.11.5 Identification of *Eimeria* species by amplification, cloning and sequencing of ITS-1/-2 regions from genomic DNA of *Eimeria* in the processed oocysts samples.

About 4 ml each of the remaining washed oocyst suspension from all the 14 samples (Nig1-14) were collected and pooled together in a single tube, centrifuged at ~750 × *g* for 10 min to pellet the oocysts and the volume of the supernatant was reduced to 1 ml. The tube was then agitated to re-suspend the pellet and 1 ml of the suspension containing about 6 x $10^6$ sporulated oocysts of the pooled samples were pelleted and disrupted by bead beating with sterile size 8 glass beads using a Mini Beadbeater-8, (Biospec Products, Bartlesville, USA) for two minutes as described earlier. Total genomic DNA was isolated from the smashed oocyst homogenate using a QIAamp DNA Tissue mini kit (Qiagen, Germany) as per the manufacturer's protocol.

PCR was used to amplify the targeted ITS-1/-2 regions from the extracted gDNA using Universal genus-specific primers (Table 3.1) in a duplicate PCR reaction mixture. The universal, genus-specific DNA primers targeting genomic ITS-1/-2 regions from *Eimeria* spp. were designed previously by aligning sequences from each region for all chicken *Eimeria* spp. using ClustalX (Schwarz *et al*., 2009). Regions of highly conserved sequence were determined from the alignment and used as priming sites. The primers are designed in the 18S rRNA (primer ITS-1) and 28S rRNA (primer ITS-2) genes, thus amplifying from the nuclear ribosomal gDNA the partial 18S rRNA, full-length ITS-1, 5.8S rRNA, and ITS-2, and the partial 28S rRNA loci (Schwarz *et al*., 2009).

The PCR was carried out in a 25.0 µl PCR reaction mixture which included 2 µl genomic DNA template, 25 pmol forward and reverse universal primer, 0.5 U *Taq* polymerase (GoTaq® DNA Polymerase kit, Promega, Madison, Wisconsin), 10mM Tris-HCl, 1.5mM MgCl2, 50mM KCl and 200 µM dNTPs. The thermal cycling condition included an initial denaturing step at 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 60 sec and a final extension at 72°C for 10 min using a Gstorm thermal cycler. The PCR products were analysed by gel electrophoresis in 1% agarose gel in 1xTris Acetate EDTA (TAE) buffer stained with a nucleic acid dye. A QIAquick gel extraction kit (Qiagen Inc.) was used to purify PCR amplicons of the anticipated size, determined by comparison with a molecular DNA ladder, and these were cloned using pGEM®-T easy (Promega) vector system kits and transformed into XL-Blue *Escherichia coli* cells. Colour screening identified recombinant clones, and 19 colonies from each PCR reaction were picked creating a panel of 38 colonies. Each of these colonies was tested for the inserts using the same PCR primers and conditions above with 1 µl overnight LB broth culture as template.

Confirmed inserts were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) with vector priming sites (M13), and sequences were obtained using an ABI 3730 genetic analyzer (Applied Biosystems). Sequence data files were verified for quality and accuracy using SequencherTM 4.8 software (Gene Codes Corporation, Ann Arbor, Michigan). Sequence identity of inserts was determined using MEGABLAST algorithm searches of the nucleotide database collection (nr/nt) via the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/).

To assess the level of genetic variability within and between *Eimeria* spp., PCR fragments amplified were cloned and sequenced such that the nucleotide reads obtained were unambiguous. Primers were trimmed from the sequences to avoid potential bias in subsequent sequence analysis.

Table 3.1: PCR primers and conditions used in the *Eimeria* species identification of the present study.

| PCR type | Species | Primer sequence 5′→3′ | Annealing temperature (°C) | Expected product size (bp) |
|---|---|---|---|---|
| Nested PCR | *Eimeria* species. | EF1[a]: AAG TTG CGT AAA TAG AGC CCT C | 55 | Variable |
| | | ER1[a]: AGA CAT CCA TTG CTG AAA G | | |
| | *E. acervulina* | EAF[a]: GGC TTG GAT GAT GTT TGC TG | 71 | 321 |
| | | EAR[a]: CGA ACG CAA TAA CAC ACG CT | | |
| | *E. brunetti* | EBF[a]: GAT CAG TTT GAG CAA ACC TTC G | 71 | 311 |
| | | EBR[a]: TGG TCT TCC GTA CGT CGG AT | | |
| | *E. maxima* | EMRA1[a]: GTG AT/AT CGT TC/TG G/AG/AA GTT TGC | 71 | 145 |
| | | EMRA2[a]: CT/AC ACC ACT CAC AAT GAG GCA C | | |
| | *E. mitis* | EMI1FA[a]: GGG TTT ATT TCC TGT CC/GT CGT CTC | 58 | 328 |
| | | EMI1RA[a]: GCA AGA GAG AAT CGG AAT GCC | | |
| | *E. necatrix* | ENF[a]: TAC ATC CCA ATC TTT GAA TCG | 61 | 383 |
| | | ENR[a]: GGC ATA CTA GCT TCG AGC AAC | | |
| | *E. praecox* | EPF[a]: CCA AGC GAT TTC ATC ATT/C GG GGA/G | 61 | 116 |
| | | EPR[a]: AAA A/GCA A/CAG CGA TTC AAG | | |
| | *E. tenella* | ETF[a]: AAT TTA GTC CAT CGC AAC CCT | 65 | 278 |
| | | ETR[a]: CGA GCG CTC TGC ATA CGA CA | | |
| ITS-2 PCR for OTUs | *E. OTU-Y* | OTU-YF[b]: CAAGAAGTACACTACCACAGC ATG | 56 | 346 |
| | | OTU-YR[b]: ACTGATTTCAGGTCTAAAACG AAT | | |
| | *E. OTU-Z* | OTU-ZF[b]: TATAGTTTCTTTTGCGCGTTGC | 56 | 147 |
| | | OTU-ZR[b]: CATATCTCTTTCATGAACGAA AGG | | |
| ITS-1/ITS-2 PCR | *Eimeria* species | ITS-1[c]: GGATGCAAAAGTCGTAACACGG | 52 | ~ 873 - ~1,010 |
| | | ITS-2[c]: TCCTCCGCTTAATAATATGC | | |

Primers source: [a]Lew *et al*., 2003; [b]Fornace *et al*., 2013; [c] Schwarz *et al*., 2009

### 3.12 Amplification, Cloning and Sequencing of the Full Length *E. tenella* AMA-1 Coding Region.

A sub-sample of 4,000 µl washed oocyst suspension containing about 4 to 5 x $10^6$ sporulated oocysts from 3 isolates (Ng 2, 3 and 8) were pelleted by centrifugation at ~750 × *g* for 10 min. The oocysts were then disrupted by bead beating with sterile size 8 glass beads using a Mini Beadbeater-8, (Biospec Products, Bartlesville, USA) for two minutes as described earlier. Total RNA were extracted from the smashed oocyst samples using Qiagen RNeasy kit and cDNA were made from the extracted RNA using Invitrogen SuperScript II kit by following the respective manufacturers instructions. Full length EtAMA-1 coding region were then amplified using the following primers (unpublished):

EtAMA1_f: ATGCGGCGGCTTTCCCCAGCTC

EtAMA1_r: CTAGTATTCCTGGTCCAGCAGC

Each PCR reaction contained 50 ng template cDNA, 50 pmol of the primers, 0.5 U Invitrogen Taq polymerase, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl and 0.2 mM dNTPs. Standard cycle parameters were 1 × (1 min at 95°C), 30 × (30 sec at 95°C, 30 sec at 56°C, 2 min at 72°C) and 1 × (10 min at 72°C). Post-amplification PCR products were resolved by agarose gel electrophoresis (1% gel in 1x TAE buffer). The PCR fragments of interest were gel excised and purified using minelute gel purification, (Qiagen), cloned using pGEM®-T Easy (Promega) vector system kits, transformed into XL1-Blue *E. coli* (Stratagene) and miniprepped (Qiagen) as described by the respective manufacturers. Candidate plasmids identified by EcoRI digestion and gel electrophoresis were sequenced (GATC Biotech) using the T7 and SP6 sequences within the T Easy plasmid and analysed using CLC Main Workbench version 5.7.1 (CLC Bio, Denmark). Ten clones were assembled for each sample for comparison for possible polymorphism.

### 3.13 Next-generation Sequencing of an *E. tenella* Field Isolate Whole Genome.

About 1µg in 60µl of the isolated genomic DNA from sample Nig5 was subjected to paired end TruseqDNA next generation whole genome sequencing using Illumina Hiseq2000 sequencer (Source BioScience Plc, Nottingham, UK) after passing quality control. For the sequence data a customized Bioinformatics pipeline was setup which consisted of four parts: adapter and quality trimming, mapping of trimmed reads against the reference *E. tenella* Houghton strain, calling for variants and building a consensus sequence from pileup. Briefly, the sequence data was adapter-and quality-trimmed using Cutadapt (version 1.3) and mapped using BWA-mem (version 0.7.5a) against Houghton UK 2007 reference strain of *E. tenella*: (Wellcome Trust Singer Institute ftp site). The resulting BAM files were sorted by coordinate and indexed for visualization with common genome browser software. Reads mapped against the reference genome were used for calling single nucleotide polymorphisms (SNPs) and small insertions or deletions (indels) present in the sample. Variant calling was carried out using Samtools (0.1.19+) and Bcftools (0.1.19-44428cd). In a final step from Samtools mpileup a consensus sequence was then built using Bcftools and Vcfutils (0.1.9.19+).

The next gen sequence datasets of the *E. tenella* isolate of this study, was compared with those generated in a related study from Wisconsin (US) and Nippon (Japan) strains of *E. tenella* against the 2007 *E. tenella* Houghton strain reference genome assembly.

### 3.14 Data Analysis

#### 3.14.1 Statistical analysis

The data collated from the prevalence studies were analysed using descriptive statistics (percentages and tabulations). Chi-square and odds ratio were used to determine the

association between the occurrence of coccidia oocysts in a farm with the farm location, flock type, age, size and management system in the study area. Values of $p<0.05$ were considered significant. Graphpad Prism statistical software version 4.0 was used for the statistical analysis of the data.

### 3.14.2 Phylogenetic analyses

Phylogenetic analyses of the *Eimeria* spp. ITS-1 + ITS-2 sequences, were performed using sequences aligned by using multiple sequence alignment software (MUSCLE) (Edgar, 2004) along with additional *Eimeria* spp. sequences obtained from GenBank. Comparable *Toxoplasma gondii* genomic sequences obtained from GenBank were also included in the alignments and used as out-group in the phylogenetic analyses. Sequences were trimmed at the same relative 5' and 3' ends, and alignments were imported to MEGA version 4 (Tamura *et al*., 2007) for phylogenetic analyses that included maximum likelihood (ML), Maximum parsimony (MP) and neighbour joining (NJ) methods. Robustness of phylogenies was assessed using bootstrap statistical tests (n = 1, 000).

### 3.14.3 Genetic diversity estimation

Estimates of the evolutionary divergence between sequences were conducted using the Maximum Composite Likelihood model conducted in MEGA4. Principal Co-ordinate Analysis (PCoA) was used to graphically illustrate the relationship among the dataset generated using Microsoft Excel plug-in software, 'GenalEx6' (http://www.anu.edu.au/BoZo/GenAlEx/) (Peakall and Smouse, 2006).

# CHAPTER FOUR

# RESULTS

## 4.1 Prevalence of Coccidia Infection in Commercial Poultry Farms in Zaria and Environs:

Out of the 110 commercial farms visited, 123 flocks were sampled consisting of 9 (7.3%), 24 (19.5%) and 90 (73.2%) from large scale, medium scale and backyard commercial farms respectively (Table 4.1).

Table 4.2 shows the prevalence of *Eimeria* infection in flocks of commercial poultry in Zaria and environs. Of the 123 flocks sampled in the study area 30% had *Eimeria* infection. Prevalence rates of 20%, 46.7%, 25%, 16.7%, 20.7%, 20% and 33.3% were observed in Giwa, Igabi, Ikara, Makarfi, Sabon Gari, Soba and Zaria Local Government Areas respectively. Although the prevalence rates varied in the different strata of the study area, there was however no significant statistical association ($\chi^2 = 7.587$, $P > 0.05$) between locations of the flocks sampled and the occurrence of the *Eimeria* infection (Table 4.2).

The occurrence of the *Eimeria* infection in the sampled flocks based on flock type is shown in Table 4.3. Of the 93 layer flocks sampled, 26.9% had *Eimeria* infection, while 40.0% of the 30 broiler flocks sampled were also infected with *Eimeria* parasite. There was no statistically significant association ($\chi^2 = 1.856$, $P > 0.05$) between the prevalence of *Eimeria* infection observed and the type of flock sampled (Table 4.3).

Table 4.1: Distribution of sampled poultry farms based on farm size.

| Farm size | Number sampled (%) | Number of flocks sampled (%) |
|---|---|---|
| Large scale commercial (> 20, 000 birds) | 3 (2.7) | 9 (7.3) |
| Medium scale commercial (5, 000 – 20, 000 birds) | 17 (15.5) | 24 (19.5) |
| Backyard commercial (200 – 5, 000) | 90 (81.8) | 90 (73.2) |
| Total | 110 | 123 |

Table 2.2: Prevalence of *Eimeria* infection among the sampled flocks based on locations.

| Flocks location | Number sampled | Number (%) positive | OR | 95% CI for OR |
|---|---|---|---|---|
| Giwa | 10 | 2 (20) | 2.00 | 0.36 to 11.23 |
| Igabi | 30 | 14 (46.7) | 0.57 | 0.20 to 1.62 |
| Ikara | 8 | 2 (25) | 1.50 | 0.26 to 8.82 |
| Makarfi | 6 | 1(16.7) | 1.83 | 0.19 to 17.86 |
| Sabon Gari | 29 | 6 (20.7) | 1.92 | 0.59 to 6.22 |
| Soba | 10 | 2 (20) | 2.00 | 0.36 to 11.23 |
| Zaria | 30 | 10 (33.3) | Reference | - |
| Total | 123 | 37 (30.1%) | | |

$\chi^2 = 7.587$, df = 6, P = 0.2699

Table 4.3: Prevalence of *Eimeria* infection in the study area according to flock type.

| Flock type | Number sampled | Number positive (%) | OR | 95% CI for OR |
|---|---|---|---|---|
| Layers | 93 | 25 (26.9) | 1.813 | 0.77 to 4.30 |
| Broilers | 30 | 12 (40.0) | Reference | - |
| Total | 123 | 37 (30.1) | | |

$\chi^2 = 1.856$, df =1, P = 0.1731

The age specific prevalence of *Eimeria* infection in the sampled flocks of commercial chickens in Zaria and environs is shown in Table 4.4. Flocks of 10 weeks old and below had 44.4% prevalence rates of infection, those within 11- 20 weeks old had 26.7% prevalence rates while those greater than 20 weeks old had prevalence rates of 21.5%. There was statistically significant association ($\chi^2 = 6.406$, $P < 0.05$) between the flock age and the occurrence of Coccidia infection in the sampled flock with bird under the age of 10 weeks being at higher risk using odd ratio (OR) (Table 4.4).

The prevalence of *Eimeria* infection in 123 sampled flocks in Zaria and environs based on flock size is shown in Table 4.5. Flocks with 500 or less birds had the highest frequency of infection (57%) followed by flocks with more than 1000 birds (26.6%). While, birds under the category of 501-1000 flock size had lesser prevalence rate of 21.1%. The frequency of occurrence of *Eimeria* infection is statistically associated ($\chi^2 = 9.162$, $P < 0.05$) with the flock size of the birds in the study area with flocks of 500 or less birds at higher risk of infection (Table 4.5).

The prevalence of *Eimeria* infection in commercial poultry in Zaria and environ based on management system is describe in Table 4.6. Birds raised on deep litter system had higher prevalence rate of 33.9% than those on battery cage system which had prevalence rate of 10%. There was statistically significant association ($\chi^2 = 4.579$, $P < 0.05$) between rate of *Eimeria* infection and the management system of the birds in the study area. Birds raised under deep litter system are statistically at higher risk of contracting *Eimeria* infection than those on battery cage system (Table 4.6).

Table 4.4: Prevalence of *Eimeria* infection among commercial poultry farms in the study area based on flock age.

| Flock age (weeks) | Number sampled | Number positive (%) | OR | 95% CI for OR |
|---|---|---|---|---|
| ≤ 10 | 43 | 19 (44.2) | 0.35 | 0.15 to 0.81* |
| 11-20 | 15 | 4 (26.7) | 0.76 | 0.21 to 2.74 |
| >20 | 65 | 14 (21.5) | Reference | - |
| Total | 123 | 14 | | |

$\chi^2 = 6.406$, df = 2, P = 0.0406, *Odd ratio (OR) value statistically significant

Table 4.5: Prevalence of *Eimeria* infection among commercial poultry farms in the study area based on flock size.

| Flock size | Number sampled | Number positive (%) | OR | 95% CI for OR |
|---|---|---|---|---|
| ≤ 50 - 500 | 21 | 12 (57.1) | 0.27 | 0.097 to 0.77* |
| 501 - 1000 | 38 | 8 (21.1) | 1.36 | 0.52 to 3.53 |
| >1000 | 64 | 17 (26.6) | Reference | - |
| Total | 123 | 37 (30.1) | | |

$\chi^2 = 9.162$, df = 2, P = 0.0102, *Odd ratio (OR) value statistically significant

Table 4.6: Prevalence of *Eimeria* infection among commercial poultry farms in the study area based on management system.

| Management system | Number sampled | Number positive (%) | OR | 95% CI for OR |
|---|---|---|---|---|
| Deep litter | 103 | 35 (33.9) | 0.22 | 0.05 to 0.98* |
| Battery cage | 20 | 2 (10.0) | Reference | - |
| Total | 123 | 37 (30.1) | | |

$\chi^2 = 4.579$, df=1, P = 0.0324, *Odd ratio (OR) value statistically significant

The physical examination of the flocks sampled indicated that all the sampled flocks were apparently healthy as they were alert and active before and during the sampling process. Also, examination of the flock record and or the flock history shows that all the sampled flocks had history of anticoccidia medication.

Out of the 37 *Eimeria* positive samples 14 were selected on the basis of having higher oocysts loads as explained earlier. The detailed flock history (location, type, age, size, management system and anticoccidia medication used) of the 14 selected *Eimeria* samples are shown in Table 4.7.

Table 4.7: Flock history of the 14 selected *Eimeria* samples of the study.

| Sample number | Flock location | Flock type | Flock age (weeks) | Flock size | Management system | Anticoccidia used |
|---|---|---|---|---|---|---|
| Nig1 | Zaria | layers | 20 | 200 | Deep litter | Amprolium |
| Nig2 | S/Gari | broilers | 7 | 400 | Deep litter | Sulfaquinoxaline, Diclazuril |
| Nig3 | Igabi | layers | 22 | 5000 | Battery cage | Amprolium, Tolturazuril |
| Nig4 | Igabi | broilers | 6 | 200 | Deep litter | Diveradine + Sulfaquinoxaline |
| Nig5 | Giwa | broilers | 9 | 500 | Deep litter | Amprolium + Sulfaquinoxaline |
| Nig6 | S/Gari | layers | 10 | 3000 | Deep litter | Amprolium, Diclazuril |
| Nig7 | Igabi | layers | 16 | 3000 | Deep litter | Amprolium + Sulfadimidine |
| Nig8 | Igabi | broilers | 4 | 500 | Deep litter | Amprolium |
| Nig9 | S/Gari | broilers | 8 | 900 | Deep litter | Amprolium + Tolturazuril |
| Nig10 | Giwa | broilers | 10 | 300 | Deep litter | Sulfaquinoxaline, Amprolium |
| Nig11 | Ikara | broilers | 6 | 300 | Deep litter | Amprolium + Sulfaquinoxaline |
| Nig12 | Zaria | layers | 52 | 1000 | Deep litter | Amprolium, Tolturazuril |
| Nig13 | Igabi | layers | 30 | 3000 | Battery cage | Diclazuril |
| Nig14 | S/Gari | broilers | 8 | 500 | Deep litter | Amprolium |

### 4.2 Occurrence and Identification of *Eimeria* species in Commercial Poultry Farms in Zaria and Environs Using Oocyst Morphometric Method

The results of the morphometric identification method of *Eimeria* species based on shape index of oocysts in the samples examined in this study revealed the presence of all the seven widely recognised *Eimeria* species of chickens in the samples representing 9 farms in the study area. *Eimeria praecox* and *E. tenella* were identified from all (100%) of the samples examined, while *E. brunetti* and *E. mitis* were seen in 8(88.9%) of the samples. *E. acervulina* and *E. necatrix* were detected in 7(77.8) of the samples while *E. maxima* was only observed in 6(66.7%) of the 9 samples (Table 4.8).

### 4.3 Occurrence and Identification of *Eimeria* species in Commercial Poultry Farms in Zaria and Environs Using Nested PCR Technique

The result of the nested PCR however indicated the presence of only four *Eimeria* species in the study samples with percentage occurrence of 100%, 66.7%, 44.4% and 33.3% for *E. tenella*, *E. mitis*, *E. acervulina* and *E. necatrix* respectively (Table 4.8; Figure 2 – 5). None of the samples was positive for *E. brunetti*, *E. maxima* and *E. paraecox*.

Table 4.8: *Eimeria* species identified from commercial poultry farms in the study area using oocysts index and nested PCR techniques.

| | Oocyst index result | | | | | | | Nested PCR result | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | E acer | E. brun | E. max | E. mit | E. nec | E. prae | E. ten | E acer | E. brun | E. max | E. mit | E. nec | E. prae | E. ten |
| Nig1 | + | + | - | + | + | + | + | + | - | - | + | + | - | + |
| Nig2 | + | + | + | + | - | + | + | + | - | - | + | + | - | + |
| Nig3 | - | + | + | + | + | + | + | - | - | - | + | - | - | + |
| Nig4 | + | + | + | + | - | + | + | - | - | - | - | - | - | + |
| Nig5 | + | + | + | - | + | + | + | - | - | - | - | - | - | + |
| Nig7 | + | + | + | + | - | + | + | + | - | - | + | - | - | + |
| Nig8 | + | + | - | + | + | + | + | - | - | - | + | - | - | + |
| Nig11 | - | + | - | + | + | + | + | - | - | - | - | - | - | + |
| Nig12 | + | - | + | + | + | + | + | + | - | - | + | + | - | + |

Key: E. acer = *Eimeria acervulina*; E. bru = *Eimeria brunetti*; E. max = *Eimeria maxima*; E. mit = *Eimeria mitis*; E. nec = *Eimeria necatrix*; E. prae = *Eimeria praecox*; E. ten = *Eimeria tenella*

+ = present; - = absent

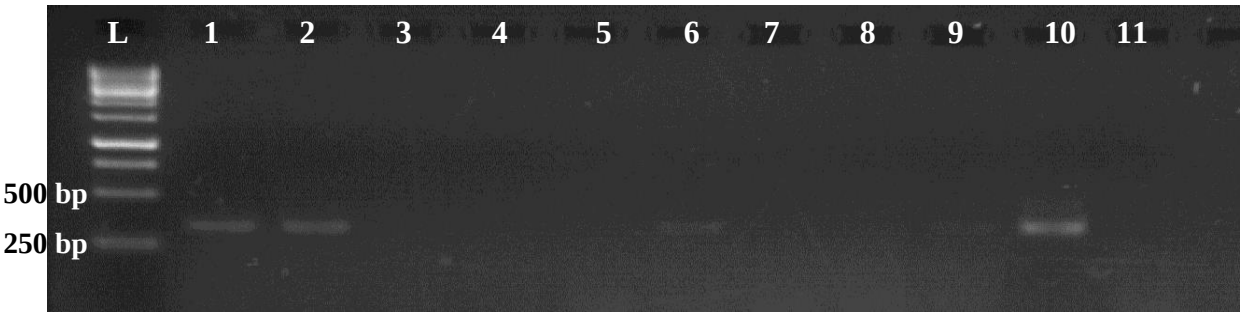



Figure 2: Nested PCR amplification of ITS-1 of *E. acervulina* (321bp) observed on 2% agarose gels stained with SaveView. Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, positive control, lane 11, negative control.

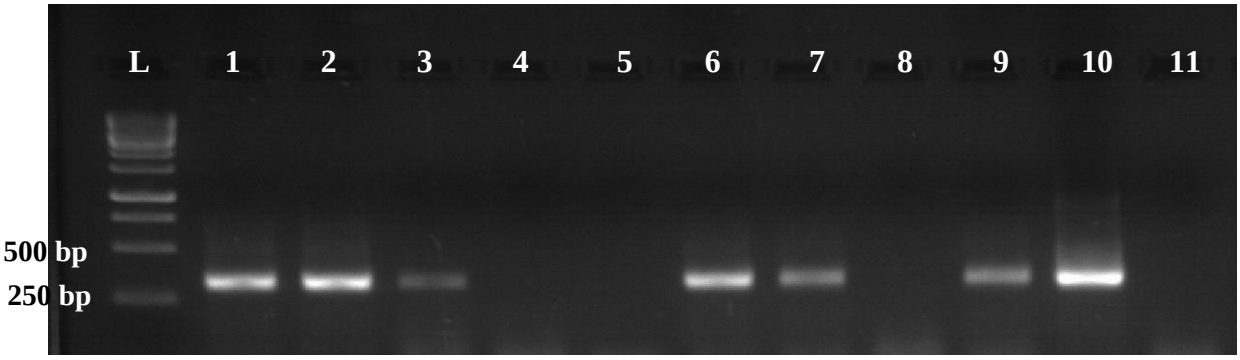



Figure 3: Nested PCR amplification of ITS-1 of *E. mitis* (328bp) observed on 2% agarose gels stained with SaveView. Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, positive control, lane 11, negative control.

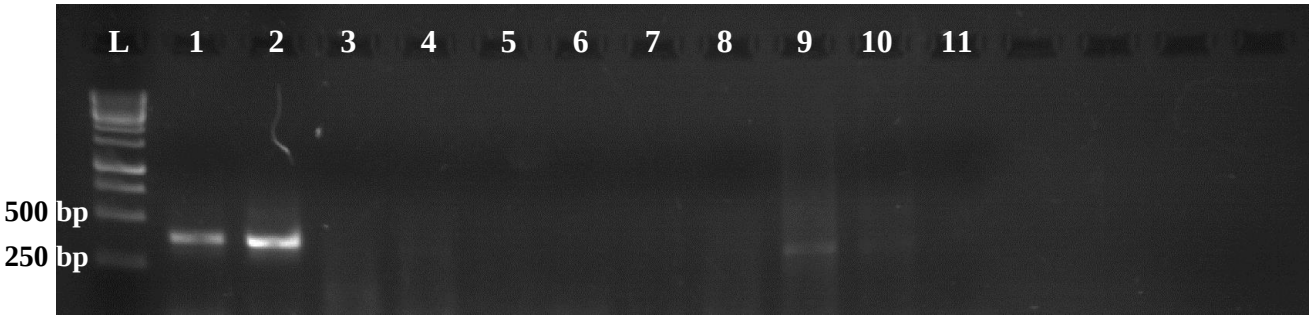



Figure 4: Nested PCR amplification of ITS-1 of *E. necatrix* (383 bp) observed on 2% agarose gels stained with SaveView.Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, positive control, lane 11, negative control.

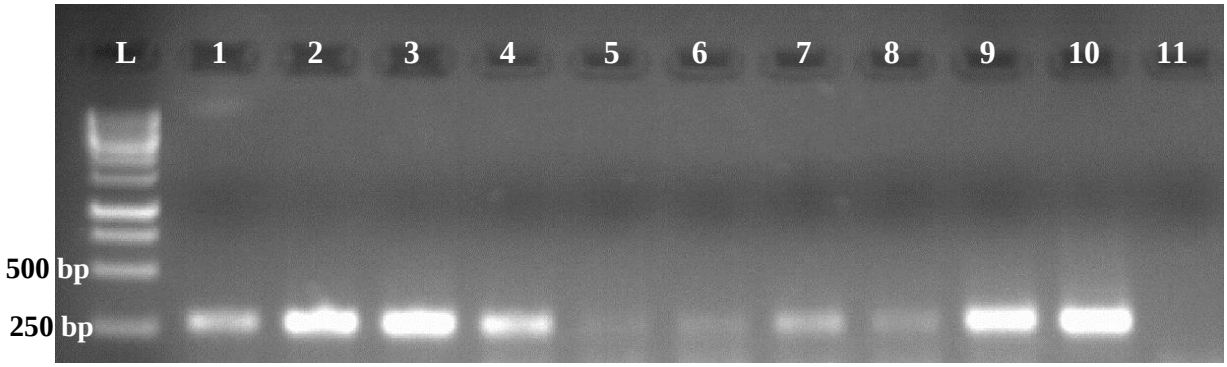



Figure 5: Nested PCR amplification of ITS-1 of *E. tenella* (278bp) observed on 2% agarose gels stained with SaveView. Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, positive control, lane 11, negative control.

### 4.4 Survey for the Presence of OTUs in Commercial Poultry Farms in Zaria and Environs Using ITS-2 PCR Technique

The screening of the samples for the presence of the OTUs revealed the presence of OTU-Y and OTU-Z in 22.2% of the 9 samples (Table 4.9; Figures 6 and 7). The occurrence of OTU-X was not determined because there were no primers available during the time of the experiment.

### 4.5 Occurrence and Identification of *Eimeria* species in Commercial Poultry Farms in Zaria and Environs Using Cloning and Sequencing of the Internal Transcribed Spacers 1 & 2 (ITS 1&2) of *Eimeria*

The electrophoretic separation of amplicons of the ITS-1 + ITS-2 PCR for the confirmation of presences of inserts in the selected panel of 38 transformed colonies showed that 34 colonies were actually carrying the cloned insert with an anticipated 2 distinct fragments sizes; a 'short' fragment of approximately 870 bps and a second 'long' fragments of approximately 1,010 bps (Figure 8). From these 29 complete sequences were obtained and the *Eimeria* species were identified from these sequences using MEGABLAST algorithm searches. The result of the search identified 18 (62.1%) of the sequences as *E. acervulina*, 3(10.3%) as *E. mitis*, 2(6.9%) sequences each as *E. maxima*, *E. tenella* and OTU-Z. While, 1(3.4%) sequence each were identified as OTU-X and OTU-Y (Table 4.10, Appendix 1).

Table 4.9: Occurrence of new operational taxonomic units (OTUs) in *Eimeria* oocysts positive litter samples from commercial poultry farms in the study area.

| Sample | OTU-Y | OTU-Z |
|---|---|---|
| Ng 1 | - | - |
| Ng 2 | - | + |
| Ng 3 | - | - |
| Ng 4 | - | - |
| Ng 5 | - | - |
| Ng 7 | + | + |
| Ng 8 | - | - |
| Ng 11 | + | - |
| Ng 12 | - | - |

Key: + = present; - = absent

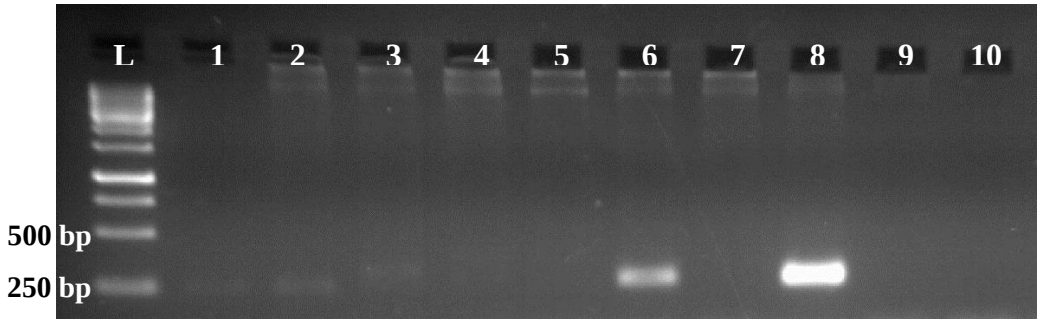



Figure 6: PCR amplification of ITS-1 of *E.* OTU-Y (346 bp) observed on 2% agarose gels stained with SaveView. Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, negative control.

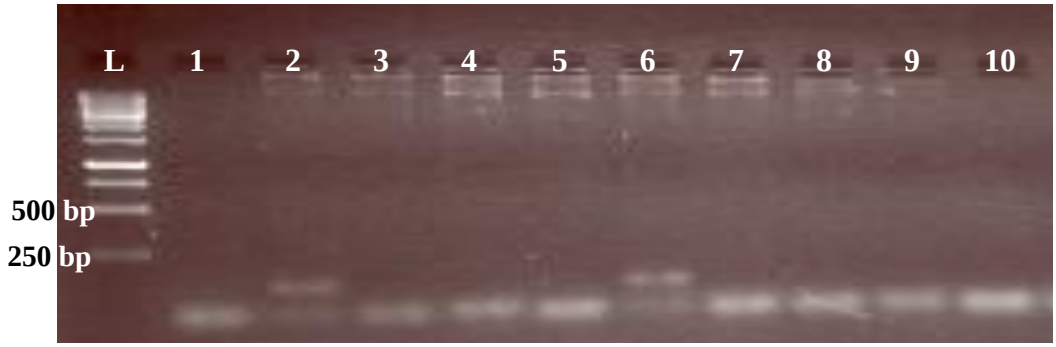



Figure 7: PCR amplification of ITS-1 of *E. OTU-Z* (147 bp) observed on 2% agarose gels stained with SaveView. Lane L, 1kb DNA ladder, lane 1-5, samples Ng1-5; lanes 6 &7, samples Ng7 & 8; lanes 8 & 9, samples Ng11 & 12 respectively. Lane 10, negative control.

Table 4.10: Result of MEGABLAST search of ITS-1&ITS-2 sequences generated from pooled *Eimeria* oocysts samples isolated from commercial poultry farms in the study area.

| *Eimeria* species | Number (%) of sequences identified |
|---|---|
| *E. acervulina* | 18(62.1) |
| *E. brunetti* | 0(0.0) |
| *E. maxima* | 2(6.9) |
| *E. mitis* | 3(10.3) |
| *E. necatrix* | 0(0.0) |
| *E. praecox* | 0(0.0) |
| *E. tenella* | 2(6.9) |
| *E.* OTU-X | 1(3.4) |
| *E.* OTU-Y | 1(3.4) |
| *E.* OTU-Z | 2(6.9) |
| Total | 29(100) |

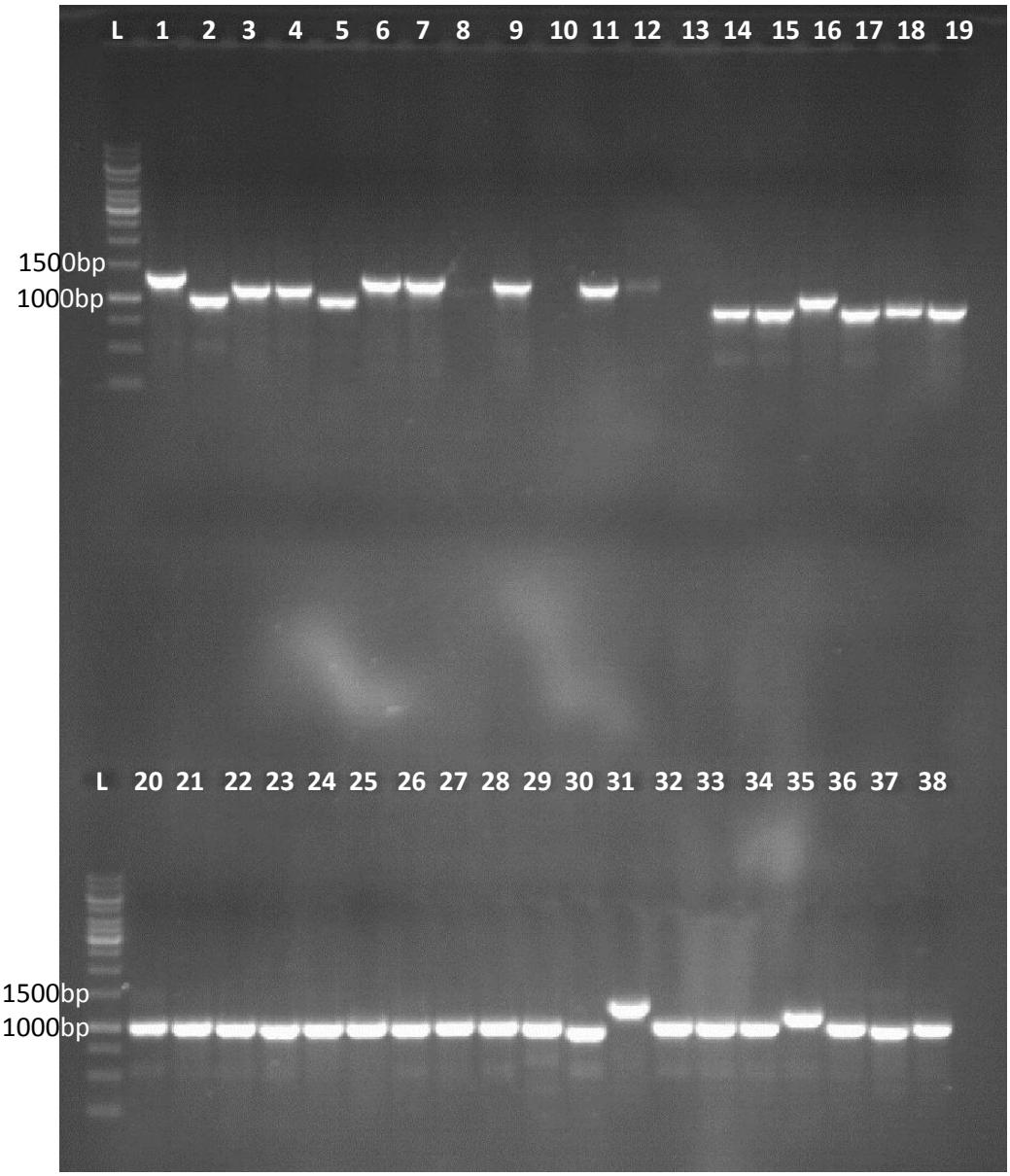



Figure 8: PCR amplification of cloned ITS-1+ ITS-2 segments of *Eimeria* species observed on 1% agarose gels stained with SaveView. Lane L, DNA ladder, lanes 1-38 anticipated incerts from selected transformed *Escherichia coli* colonies.

### 4.6 Determination of Genetic Diversity in the Internal Transcribed Spacers 1 & 2 (ITS 1&2) Sequences of *Eimeria* species Isolated from Zaria and its Environs Using Phylogenetic and Principal Coordinate Analyses.

Phylogenetic analysis of the ITS-1 + ITS-2 sequences of the *Eimeria* species from this study and from sequences of reference strains obtained from different parts of the world is shown in Figures 9 - 11. Figures 9, 10 and 11 show the results of the ML, MP and NJ analyses respectively. Sequence from *Toxoplasma gondii* was used as the out group. The phylogenetic tree constructed from the ITS-1 + ITS-2 sequences of the *Eimeria* species resulted in 11 well-defined clades with strong bootstrap support (87-99%). *Eimeria acervulina*, *E. brunetti*, *E. necatrix*, *E. praecox*, *E. tenella* as well OTU-Z each formed a separate clade. *E. maxima* basal clade resulted into three well-supported clades; two *E. maxima* clades generated in accordance to the long and short sequences form and a single clade of the OTU-X sequences. The *E. maxima* short form and the OTU-X sequences formed a sister relationship with a 99% bootstrap support. *E. mitis* sequences fell into two well supported clades, also according to the long and short sequence forms (Figures 9, 10 and 11). These phylogenetic relationships appear to be maintained in the ML, MP and NJ analyses used in this study with broadly comparable topologies for all. The Nigerian isolates of *E. maxima* and *E. mitis* fell within their respective long form and short form clades respectively. However, the phylogenetic distribution of the *Eimeria* species generally, seemed not to depend on the geographic origin of a particular isolate within clades.

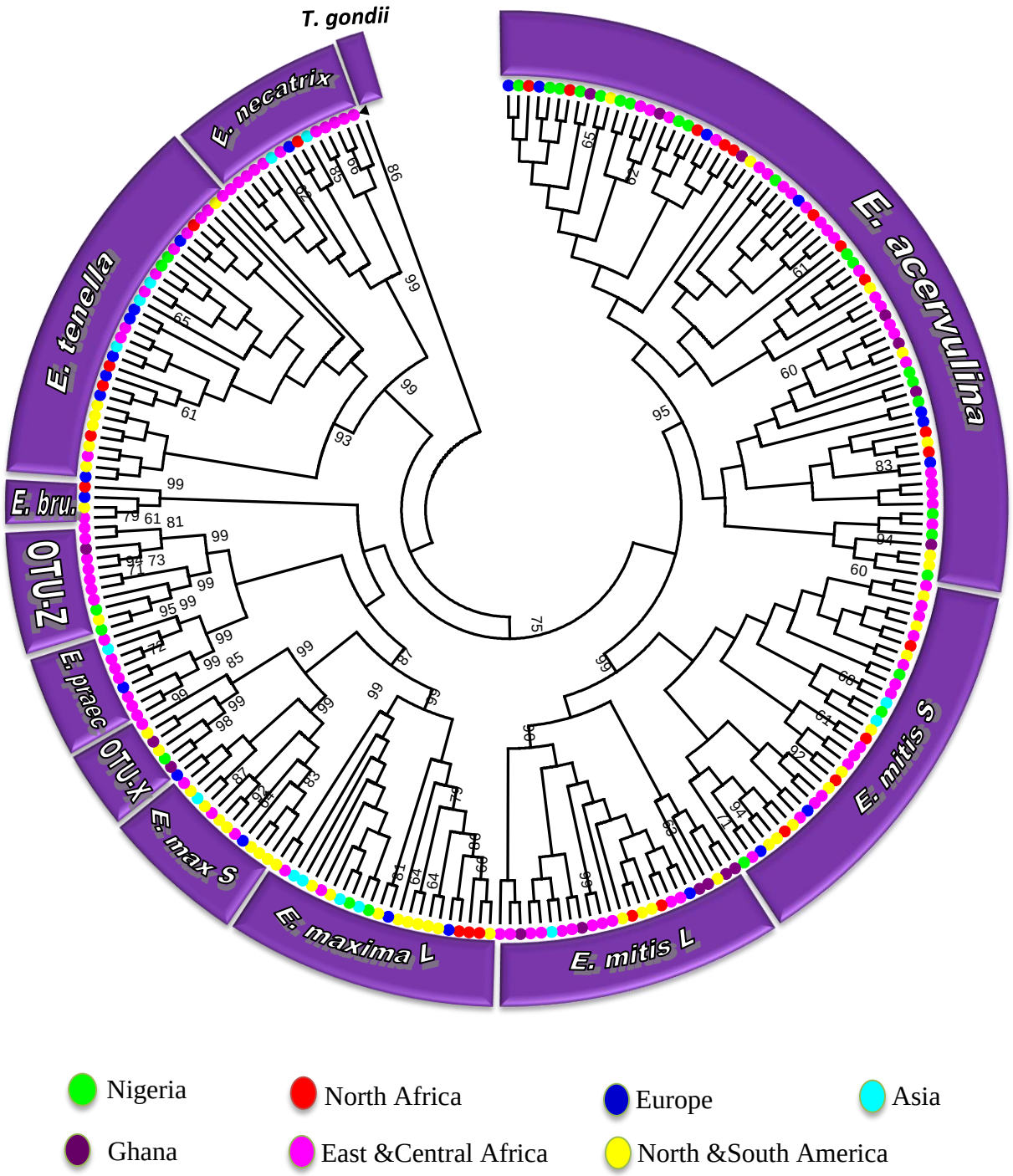



Figure 9: Phylogeny of *Eimeria* species internal transcribed spacer 1&2 (ITS-1/ITS-2) sequences of Nigerian *Eimeria* isolates and those obtained from other parts of the world produced using Tamura-Nei model Maximum Likelihood (ML) phylogeny using default parameters with 500 bootstrap replication, constructed in Mega6.1.

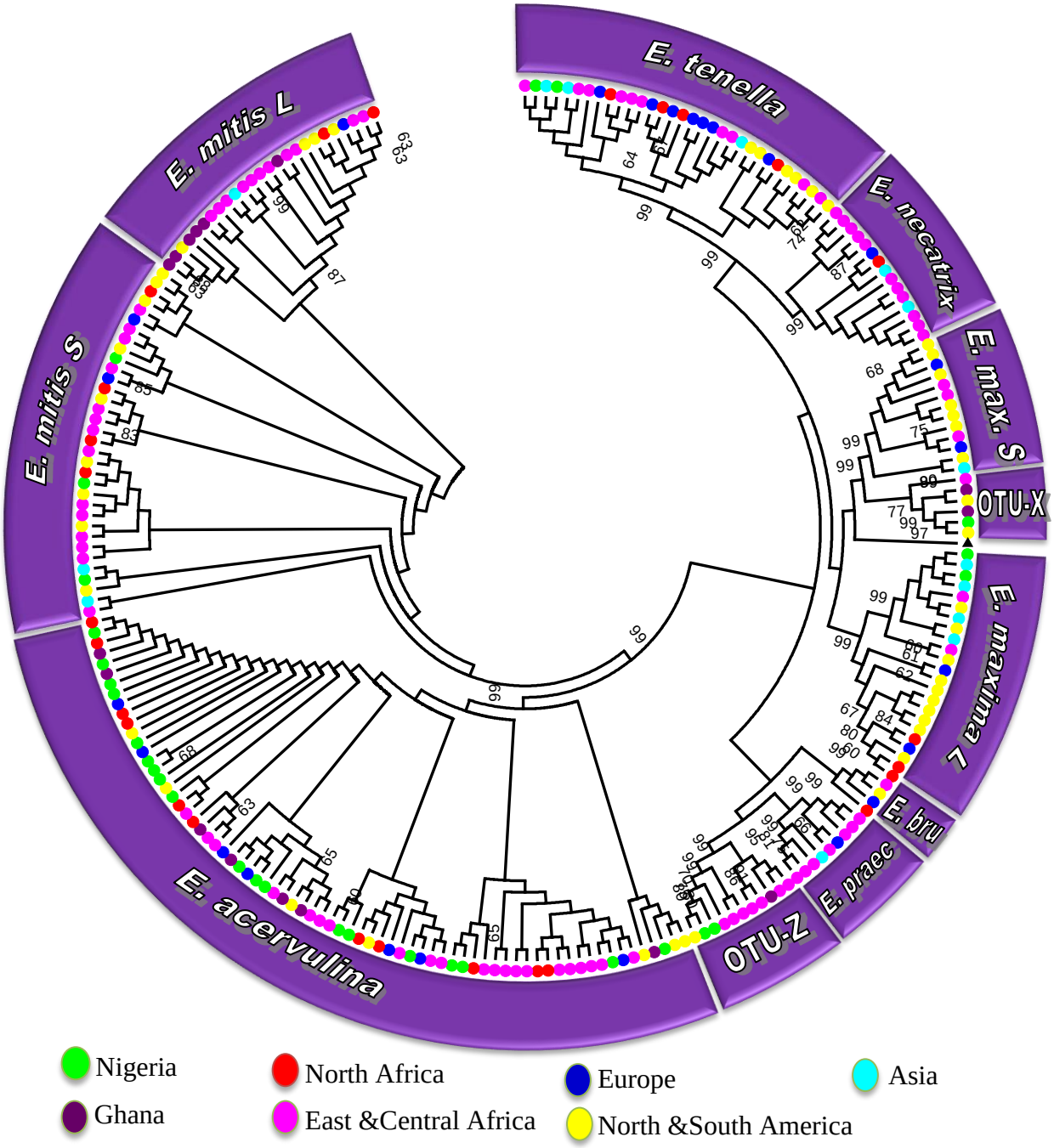



Figure 10: Phylogeny of *Eimeria* species internal transcribed spacer 1&2 (ITS-1/ITS-2) sequences of Nigerian *Eimeria* isolates and those obtained from other parts of the world produced using Tamura-Nei model Neiboughhood joining (NJ) phylogeny using default parameters with 500 bootstrap replication, constructed in Mega6.1.

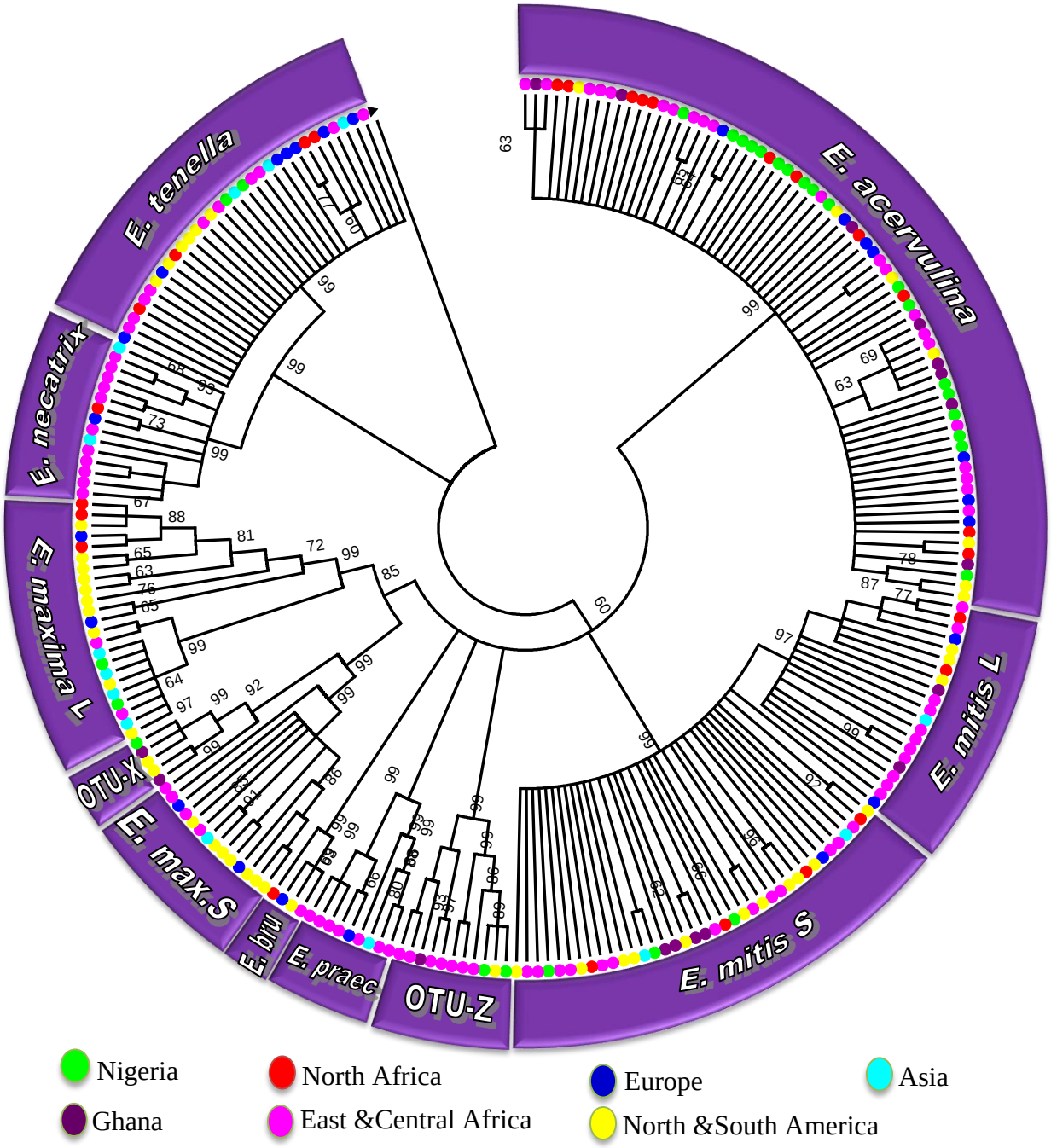



Figure 11: Phylogeny of *Eimeria* species internal transcribed spacer 1&2 (ITS-1/ITS-2) sequences of Nigerian *Eimeria* isolates and those obtained from other parts of the world produced using Kimura-2 parameter model Maximum Parsimony (MP) phylogeny using default parameters with 500 bootstrap replication, constructed in Mega6.1.

Figure 12 is the graphical illustration of Principal Co-ordinate Analysis (PCoA) of the genetic variation among the ITS-1 + ITS-2 sequences of chicken *Eimeria* species generated from this study and from reference strains from different parts of the world. Isolates of the same species clustered closely indicating genetic similarity. *Eimeria acervulina*, *E. mitis* as well as *E. necatrix* and *E. tenella* showed a higher level of genetic differences among the species which infect the chicken by forming distinct clusters. However, *E. necatrix* and *E. tenella* showed less genetic divergence by clustering close to one another. Similarly, short and long forms of *E. maxima, E. praecox*, *E. brunetti*, OTU-X and OTU-Z clustered close to each other at the middle of the graph, indicating a closer genetic relatedness among their ITS-1 + ITS-2 sequences. The overall interspecific mean diversity was 0.508±0.101.

Expanded graphical illustration of the PCoA of the mid-clustered *Eimeria* species of Figure 12 is shown in Figure 13. Here the overall mean diversity was 0.219±0.069. *Eimeria* OTU-Z clustered separately at the top left corner of the plot. Similarly, the *E. maxima* long form clustered separately at the left bottom corner of the graph. However, *E. praecox* and *E. brunetti* clustered closely at the middle of the plotted area. *E. maxima* short form and *Eimeria* OTU-X also formed a close association at the bottom left corner of the graph.

Figures 14 and 15 are the PCoA graphs for *E. acervulina* and *E. tenella* showing intraspecific genetic variation within the ITS-1 + ITS-2 sequences of different isolates. The overall mean diversity for these species was 0.019±0.002 and 0.200±0.012 for *E. acervulina* and *E. tenella* respectively. In both species, the majority of isolates clustered within the same region of the graph with only few genetically divergent outlier isolates

situated away from the main clusters. No clear evidence of allopatric selection was observed based on the distribution of the isolates in both graphs.

### 4.7 Determination of Genetic Diversity in the AMA-1 Gene of *Eimeria tenella* Isolates Using Principal Co-ordinate Analysis (PCoA).

Figure 16 is the graphical illustration of the PCoA showing genetic diversity in the AMA-1 cDNA sequences of 10 clones each from three Nigerian field samples (Nig1, Nig2 and Nig3) of *E. tenella*. Complimentary DNA of AMA-1 of reference *E. tenella* strains from United Kingdom, India, China and Japan were also used in the analysis for comparison with the Nigerian isolates. Three AMA-1 allelic types were obtained from the Nigerian samples with all clones from two samples (Nig1 and Nig3) having similar AMA-1 alleles. While clones from the third sample (Nig2) included two distinct AMA-1 allelic types, one of which is closely related to allele from the Japanese *E. tenella* strain. Strains from different countries showed distinct allelic types. The overall mean diversity of the AMA-1 allelic types was 0.004± 0.001.

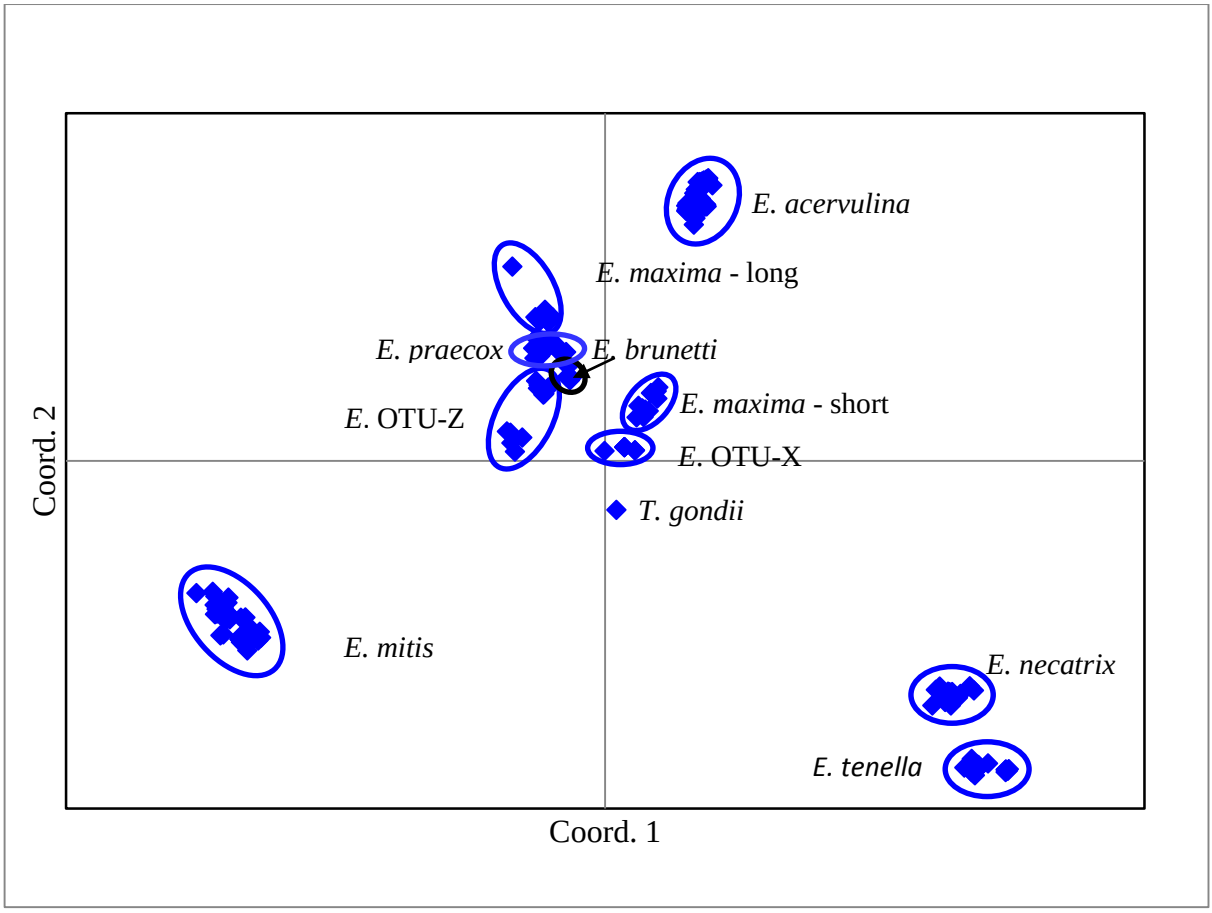



Figure 12: Principal Co-ordinate Analysis (PCoA) showing genetic variation among the ITS-1 + ITS-2 sequences of chicken *Eimeria* species from Nigeria and those obtained from other parts of the world with overall mean diversity of 0.508±0.

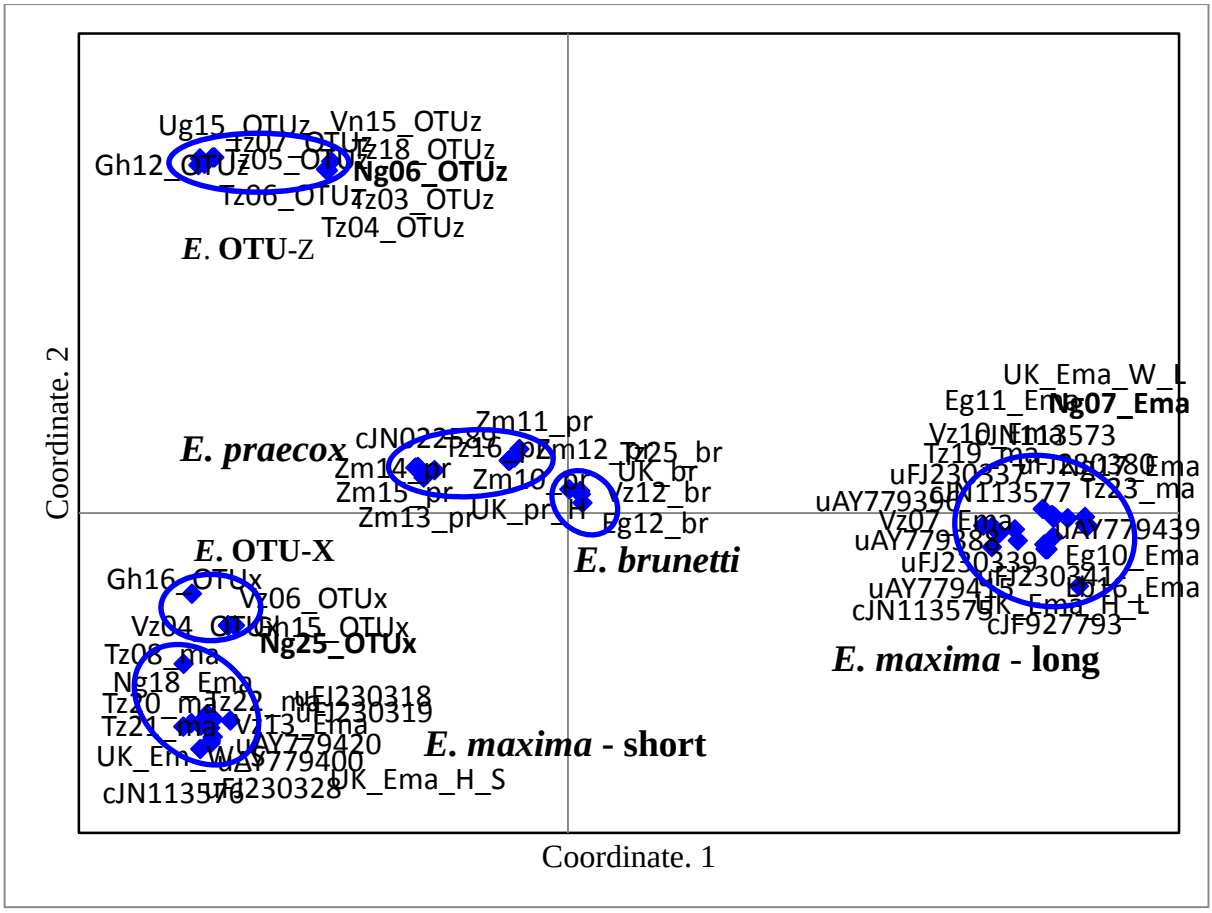



Figure 13: Principal Co-ordinate Analysis (PCoA) showing genetic variation among the ITS-1 + ITS-2 sequences of the mid-clustered *Eimeria* species obtained from Nigeria and other parts of the world in Figure 12 with overall mean diversity.

Key:
The first two letters of each sequence identifier indicate the country of origin (except for the retrieved reference strains which use u for USA and c for China.

c = China, Eg = Egypt, Gh = Ghana, Ng = Nigeria, Tz = Tanzaniz, Ug = Uganda,
u = USA, UK = United Kingdom, Vz = Venezuela and Zm = Zambia.

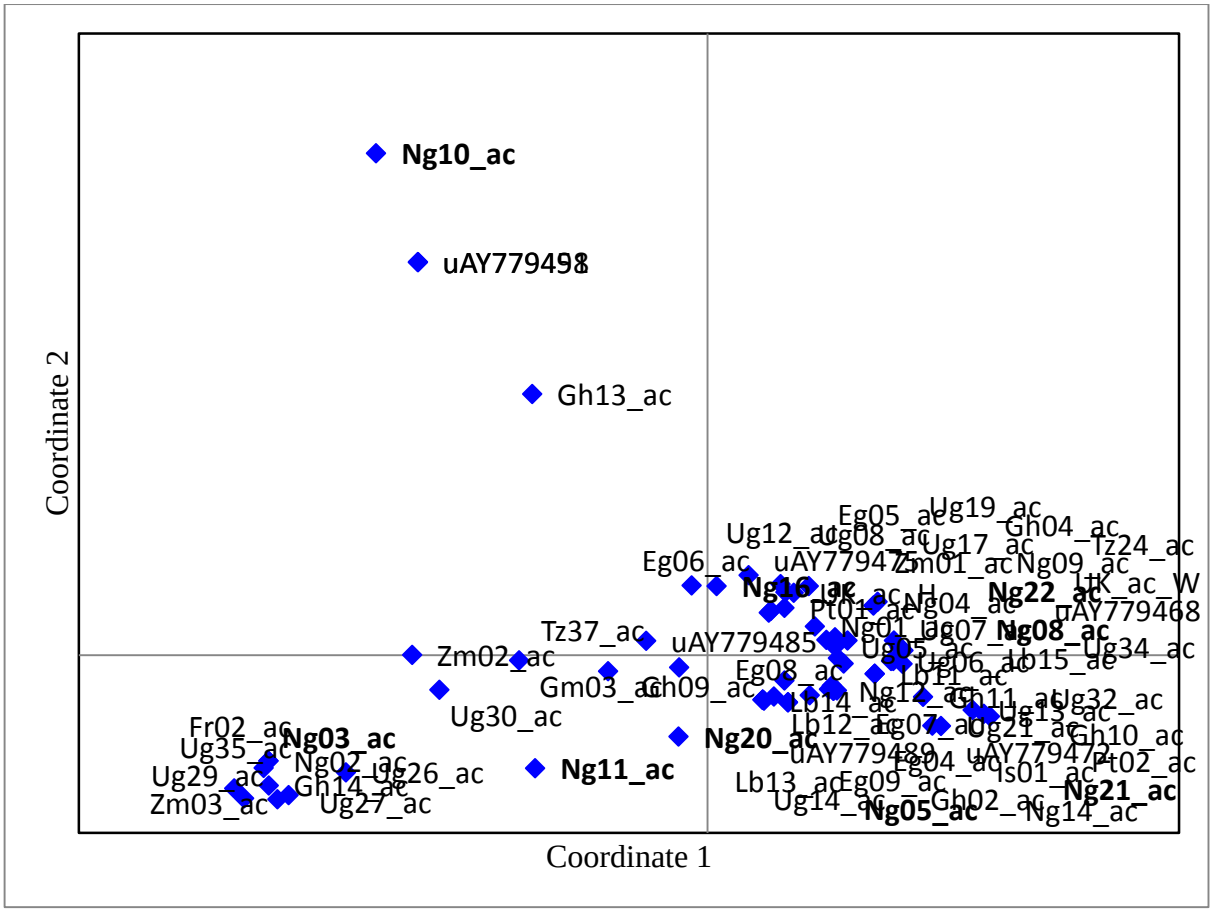



Figure 14: Principal Co-ordinate Analysis (PCoA) showing intra-specific genetic variation within the ITS-1 + ITS-2 sequences of different isolates of *E. acervulina* obtained from Nigeria and other parts of the world with overall mean diversity of 0.019±0.002.

Key:

The first two letters of each sequence identifier indicate the country of origin (except for the retrieved reference strains which use u for USA and c for China.

c = China, Eg = Egypt, Fr = France, Gh = Ghana, Gm = Germany Lb = Libya, Ng = Nigeria, Tz = Tanzaniz, Ug = Uganda, u = USA, UK = United Kingdom, Vz = Venezuela and Zm = Zambia.

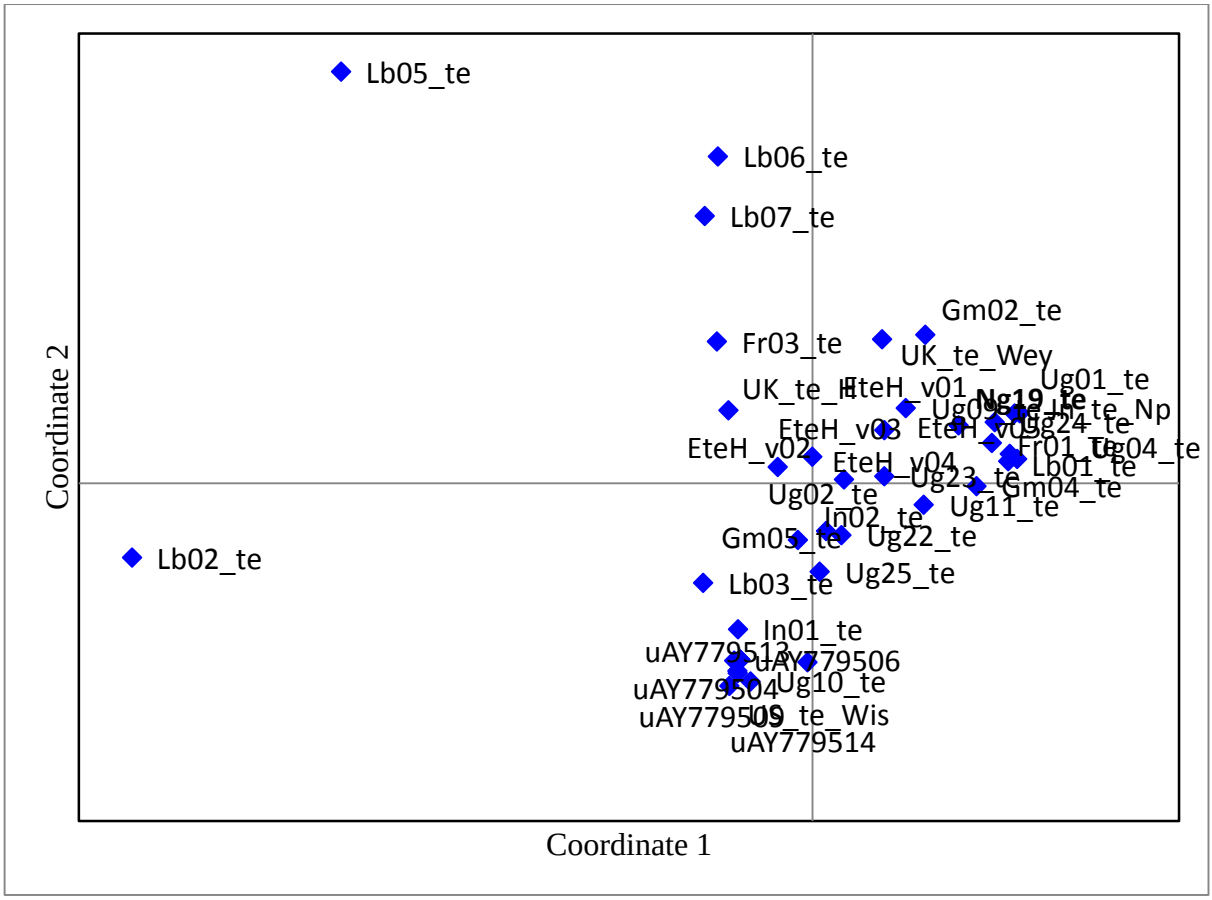



Figure 15: Principal Co-ordinate Analysis (PCoA) showing intra-specific genetic variation within the ITS-1 + ITS-2 sequences of different isolates of *E. tenella* obtained from Nigeria and other parts of the world with overall mean diversity of 0.200±0.012.

Key:
The first two letters of each sequence identifier indicate the country of origin (except for the retrieved reference strains which use u for USA and c for China.

c = China, Eg = Eagypt, Fr = France, Gh = Ghana, Gm = Germany Lb = Libya, Ng = Nigeria, Tz = Tanzaniz, Ug = Uganda, u = USA, UK = United Kingdom, Vz = Venezuela and Zm = Zambia.

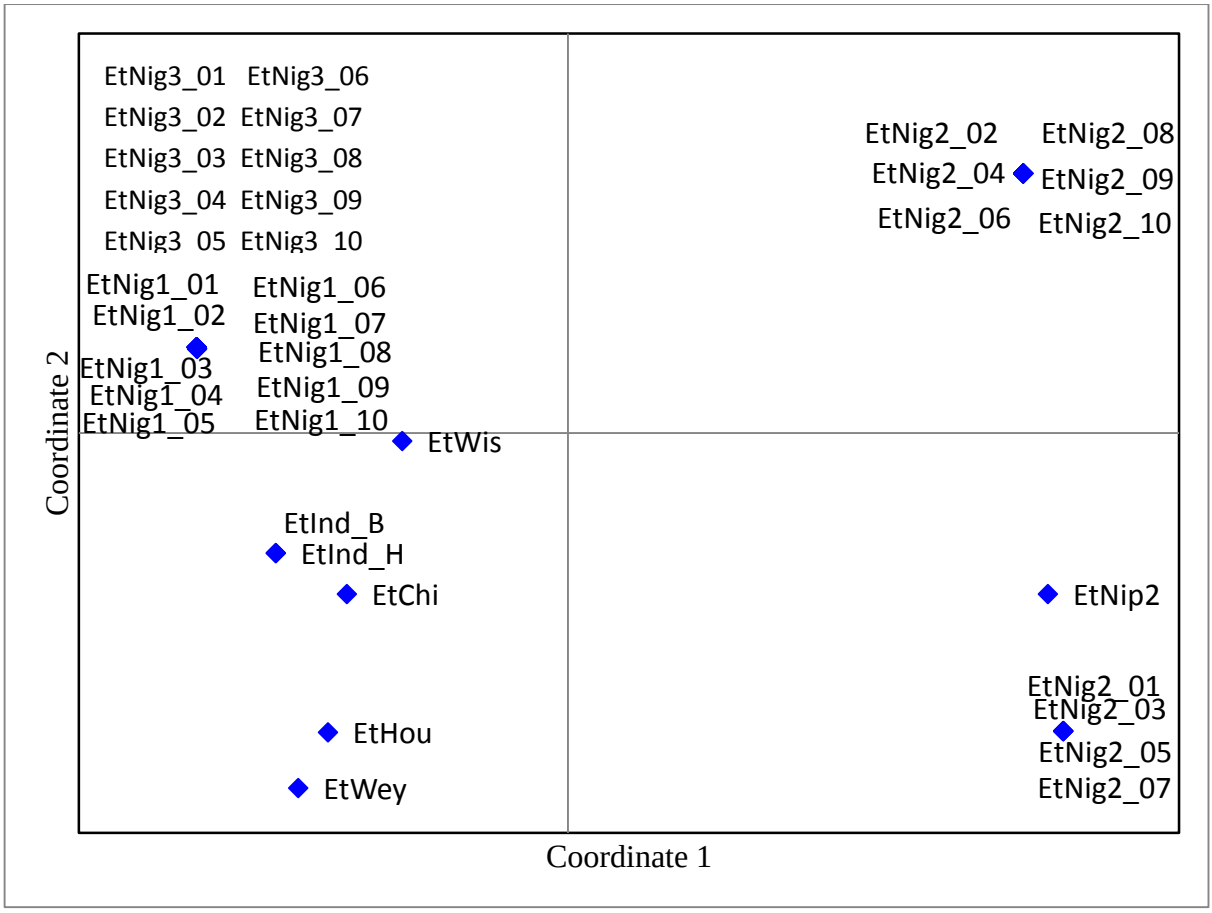



Figure 16: Principal Co-ordinate Analysis (PCoA) showing genetic diversity in the AMA-1 cDNA sequences of 10 clones each from three Nigerian field samples (Nig1, Nig2 and Nig3) and reference strains of *Eimeria tenella* with overall mean diversity of 0.004± 0.001.

Figure 17 is a PCoA graph illustrating the diversity among the translated amino acid sequences obtained from the AMA-1 cDNA. The result revealed three distinct isoforms from the Nigerian *E. tenella* field samples. The first isoform was derived from the AMA-1 cDNA of two of the samples (Nig1 and Nig3) and that of a strain from India (EtInd). This isoform is very similar (difference of one nucleotide) to the isoform derived from the AMA-1 cDNA of the Houghton strain from the UK. The two other isoforms were obtained from the AMA-1 cDNA sequences of clones from the third Nigerian sample (Nig2); one of the isoforms being the same as that derived from the Japanese strain (EtNip2). Strains from China (Et_Chin), UK (EtWey) and US (EtWis) formed distinct isoforms. The overall mean diversity among the different isoforms was 0.006± 0.002.

The specific areas of amino acid diversity among the different observed isoforms of AMA-1 of *E. tenella* isolates from the present study is shown in Table 4.11. A total of ten biallelic polymorphic residues were identified with their respective dominant and minor amino acids in each position identified in the table. With the exception of position 49 and 462, the polymorphic regions appeared to cluster within two regions (283 -293 and 332 – 386) of the protein.

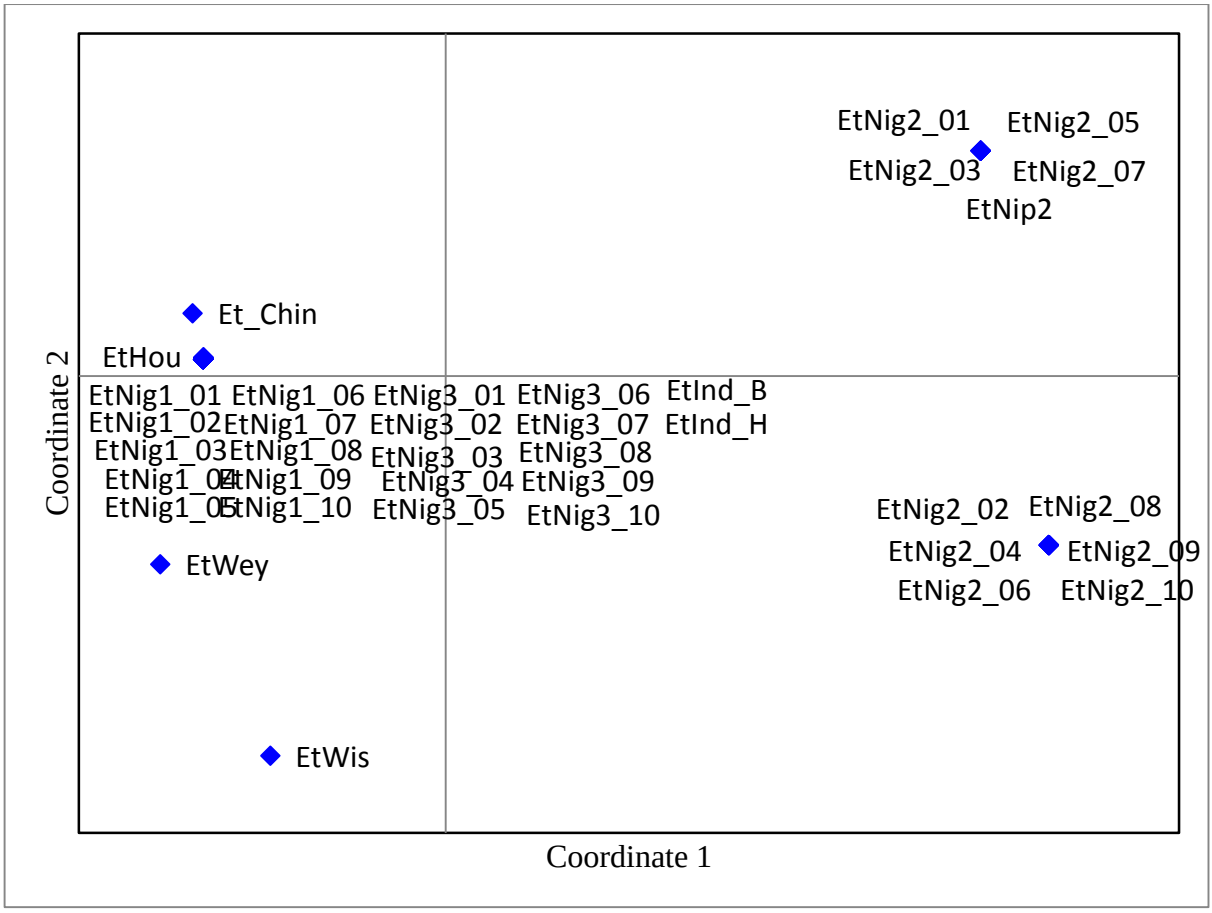



Figure 17: Principal Co-ordinate Analysis (PCoA) showing genetic diversity in the amino acid sequences obtained from AMA-1 cDNA sequences of 10 clones each of three Nigerian field samples (Nig1, Nig2 and Nig3) and reference strains of *Eimeria tenella* with an overall mean diversity of 0.006± 0.002.

Table 4.11: Positions of amino acid polymorphism among the AMA-1 isoforms from Nigerian isolates of *E. tenella* and that of strains from UK, USA and Asia.

| Amino acid position | Dominant amino acids | Minor amino acids |
|---|---|---|
| 49 | Val | Ala |
| 283 | Val | Gly |
| 289 | Cys | Gly |
| 292 | Gln | Gly |
| 293 | Arg | Gly |
| 332 | Thr | Lys |
| 333 | Ser | Arg |
| 378 | Gln | Arg |
| 386 | Val | Glu |
| 462 | - | Leu |

### 4.8 Determination of Genetic Diversity in the Whole Genome Sequence of Nigerian Isolate of *E. tenella*.

Comparison of next generation sequence dataset of the *E. tenella* isolate from the present study (Nig5), a strain from US (Wisconsin) and Japan (Nippon) from a related project were compared with the reference sequence assembly of *E. tenella* Houghton strain (Table 12). The reference assembly used was the 2007 assembly of the *E. tenella* Houghton strain (https://www.sanger.ac.uk/resources/downloads/protozoa/eimeria-tenella.html), which included 41.59 Mb of sequence. The consensus assemblies for the strains all included in excess of 98.7% coverage, with the lowest coverage for the Nigerian isolate. The lowest number of single nucleotide polymorphisms (SNPs) was observed between the reference and the US strains. The Japanese strain had more SNPs with even more for the Nigerian isolate (Table 4.12).

Table 4.12: Comparison of *E. tenella* Houghton strain (UK) reference genome with genomes of isolates from US, Japan and Nigeria.

| *E. tenella* strain | Sequence Assemblies | | |
|---|---|---|---|
| | Size (Mb) | % of Houghton | No. of SNPs |
| *Houghton (UK) | 41.59 | - | - |
| Wisconsin (US) | 41.29 | 99.28 | 30420 |
| Nippon (Japan) | 41.31 | 99.34 | 35903 |
| Nig5 (Nigeria) | 41.06 | 98.74 | 47093 |

*Reference assembly.

# CHAPTER FIVE

# DISCUSSION

The detection of *Eimeria* oocysts in 30% of the farms sampled is an indication that coccidiosis is a widely occurring disease in the study area. The prevalence is similar to that previously reported among slaughtered chickens in the study area (Jatau *et al*., 2012) and in the southern part of Nigeria (Etuk *et al*., 2004). This agrees with the report of Adene and Oluleye (2004) which states that coccidiosis is the major parasitic disease of Poultry in Nigeria. Although different prevalence rates of *Eimeria* infection were observed at the different strata of the study area, it was however, not statistically significant. This observation concurred with report of Luu *et al*. (2013) that also observed no evidence of difference in the prevalence of *Eimeria* oocysts in faecal samples of chickens among different studied regions in Ethiopia. This could be due to the cosmopolitan nature of chicken *Eimeria* species (Taylor *et al*., 2007; Blake and Tomley, 2014).

In the present study, the observed prevalence of *Eimeria* infection does not seems to be significantly associated statistically to the type of the sampled flocks (layers and broilers) which disagrees with reports of Etuk *et al*. (2004) and Jatau *et al*. (2012) who observed significantly higher frequencies of coccidia infection in layers than in broilers. According to McDougald and Reid (1997), breeder and layer pullets are usually at greater risk of infection by coccidia because they are kept on litter for far longer period than broilers. The discrepancies in the results could be based on the fact that in this study, fewer samples were studied than in the above previous reports.

The age specific rate of the infection was observed to be significantly higher in younger flocks of 10 weeks old or less, as also observed by many authors in previous studies (Majaro, 2001; McDougald, 2003; Shirley *et al*., 2005; Conway and McKenzie, 2007; Taylor *et al*., 2007). Outbreak of clinical coccidiosis was reported in birds as young as 5-day old in Nigeria (Musa *et al*, 2010). Immunity to avian coccidiosis under natural condition usually increases with age (Chapman, 1997; Uza *et al.*, 2001). This perhaps explains the low frequencies of infection observed in the older birds in this study, which usually develop strong resistance to coccidiosis due to the naturally acquired immunity induced by exposure to small number of oocysts from the environment, otherwise, unexposed adult birds remain highly susceptible to all *Eimeria* species (Conway and McKenzie, 2007; Taylor *et al*., 2007). This perhaps could be the reasons why oocysts were also isolated from older flocks.

Higher frequency of infection with *Eimeria* species was observed in birds within the flock size of 500 or less. This disagrees with previous reports that coccidiosis is common to farms with large flock size (McDougald, 2003; Taylor *et al*., 2007). This is because rearing of thousands of birds on deep litter may result in a tremendous build-up of the oocyst population especially when hygienic practice is compromised (Adene and Oluleye, 2004; Taylor *et al*., 2007). The significantly higher prevalence observed in the small flock sizes in the present study might be due to the fact that most farmers that keep small number of birds, raised such birds in small and poorly ventilated houses with poor management and lack of veterinary care. Farms with large number of birds are usually characterized by good biosecurity practices and constant use of prophylactic anticoccidial drugs. This might explain the reason for the low prevalence of infection observed in large flocks relative to the smaller flocks in this study.

Birds raised on deep litter system were observed to have significantly higher prevalence of *Eimeria* infection than those managed in battery cages. This observation concurs with previous reports that coccidiosis is common to birds raised on deep litter (Methusela *et al*., 2002; Adene and Oluleye, 2004; Taylor *et al*., 2007; Blake and Tomley, 2014). This is because rearing birds on deep litter favours accumulation of oocysts and offers optimal conditions of temperature and humidity for oocyst sporulation with the birds having direct contact with the contaminated litter materials. In cage system, there is minimal or no contact between the birds and their faeces, thus transmission would be minimal and might be the major reason for the low frequencies of infection observed in birds reared in battery cages in this study. Other authors have similarly reported low cases of coccidiosis in birds raised in cages (Reid, 1990; Lawal *et al*., 2001; Etuk *et al*., 2004). It was presumed that the possible causes of infection in caged birds are linked to human beings, flies and other insects that mechanically introduced the oocyst to the birds in cages as well as from contaminated feed during processing by the manufacturers.

All the sampled flocks appeared apparently normal based on the physical assessment of the flocks despite the detection of *Eimeria* oocysts in the faeces indicating the birds might have developed immunity to the trickle infections acquired from the environment which enable them to maintain the state of balance to the infection in the form of sub-clinical disease (Blake and Tomley, 2004). The implication of this finding is that birds' performance in the infected flocks may be affected negatively with the farmers not aware of the flocks' problem. Haug *et al*. (2008a) and Jatau *et al*. (2014) reported significant decresased in commercial performance in the form of reduced egg production and weight gain respectively, in birds with subclinical coccidiosis. More so, the reproductive capacity and pathogenicity of the *Eimeria* species suggests that, the mere presence of the

parasite in a flock indicates an immediate risk of outbreak of clinical coccidiosis. The fact that all the sampled flocks had history of anticoccidia prophylactic treatment suggests that the isolated *Eimeria* species may have some degree of tolerance or resistance to the drugs. Capacity for the development of resistance by *Eimeria* to all available anticoccidial drugs have been established (Chapman, 1998; Abbas *et al*., 2011). According to Chapman (1999) most anticoccidial drugs used in the field today only partially suppress parasite development thereby ameliorating the clinical manifestation of the disease. This may contribute to the observed wide spread subclinical coccidiosis in the sampled flocks.

The present study is the first to report the occurrence of *Eimeria* species at farm levels in Northern Nigeria using molecular techniques. It is also the first report of the occurrence of the genetically variant *Eimeria* species (OTU- X, OTU-Y and OTU-Z) in chickens in Nigeria. Indeed prior to this study OTU-Y was only reported in Australia (Cantacessi *et al*; 2008, Fornace *et al*: 2013). Multiple *Eimeria* species were detected in most of the positive farms which indicates a complex species population structure in the study area and is an indication of potential economic impact on poultry production in the study area and in Nigeria at large. This observation concurs with previous reports that under natural condition, concurrent infection of chickens with multiple *Eimeria* species is very common (McDougald, 2003; Haug *et al*., 2008b). Thus, this ubiquity and complex species population of chicken *Eimeria* might precludes eradication as a practical option for control especially in intensively managed poultry farms.

The genetically variant *Eimeria* species of chickens (OTUs) were only previously identified in Australia (Cantacessi *et al*., 2008). However, recent report by Fornace *et al*.

(2013) and the result of the present study suggest that these species are widespread in Africa. Reliance on imported day old chicks or parent stocks in most African poultry industries might have contributed to the introduction of these species to Africa. While there is presently no detailed information on the biology of these new OTUs, preliminary infection experiments and molecular characterization of each of them indicated clear distinctiveness in biology from the seven currently recognized *Eimeria* species of chickens (Gasser *et al*., 2005). The phylogenetic analysis of the ITS sequences of these new OTUs in relation to the recognised *Eimeria* species of chickens suggested that OTU-X and OTU-Y represent a population variants of *E. maxima* and *E. brunetti* respectively, while OTU-Z represent cryptic species of *Eimeria* (Cantacessi *et al*., 2008; Schwarz *et al*., 2009; Fornace *et al*., 2013). This hypothesis was also confirmed in the present study and is an indication that the new OTUs could as well be pathogenic to chickens owing to their close relationship with the known pathogenic species. Report by Morris *et al*. (2007) suggested that these OTUs could be resistant to anticoccidia drugs that are effective against the common species because they observed sudden predominance of OTU-Y in persistent coccidiosis problem where an anticoccidial drug (Baycox1, Bayer) was used. The fact that these parasites were isolated from farms with history of anticoccidial prophylaxis also suggest that they might be resistant to the anticoccidial drugs used. More so, with the species-specific nature of host immunity against *Eimeria* species, there is possibility that the available chicken coccidia live vaccines may not protect against these OTUs, an important consideration now that they seem to be widespread in African poultry industry.

In the present study, the morphometric method of identification of *Eimeria* species seems to be very sensitive as it was able to identify all the seven widely recognised *Eimeria*

species of chicken in the samples. This concurs with previous reports of morphometric studies which showed that all *Eimeria* species of chicken can be identified using oocyst morphometry (Terra *et al*., 2001; Luchese *et al*., 2007; Haug *et al*., 2008b; Carvalho *et al*., 2011; Jatau, *et al.*, 2012). Nevertheless, this technique has limitations to be used alone in making accurate diagnosis of *Eimeria* species. This is because the measurements of the oocysts may undergo variations due to changes in metabolism of the parasites or the birds (López *et al*., 2007). Most importantly, with the exception of *E. maxima* that has the largest oocyst, the average range of oocysts dimension of most other chicken *Eimeria* species overlap leading to misleading conclusions regarding speciation using oocysts morphometric techniques (Eckert *et al*., 1995; Woods *et al*., 2000a; Haug *et al*., 2008b; Sun *et al*., 2009; Carvalho *et al*., 2011).

The use of molecular techniques is the most specific and efficient way of diagnosing *Eimeria* infection especially when it involve several species occurring concurrently (Fernandez *et al*., 2003; Morris and Gasser, 2006; Haug *et al*., 2007; Schwarz *et al*., 2009; Vrba *et al*., 2010). Based on the results of the two molecular methods employed in this study *Eimeria acervulina, E. maxima, E. mitis, E. necatrix, E. tenella* as well as the three new operational taxonomic units (OTU-X, OTU-Y and OTU-Z) are the common species of *Eimeria* circulating in the commercial poultry farms in the study area. *Eimeria brunetti* and *E. praecox* were not detected by the molecular methods, a finding that disagrees with the report of Ogedengbe (2008) that identified all the seven recognized *Eimeria* species of chicken from clinic samples in Vom, North central Nigeria using multiplex PCR; and a recent report of Fornace *et al*. (2013) where they identified the presence of all the seven *Eimeria* species of chicken and two genetic variants (OTU-X and OTU-Z) in small-scale commercial poultry farms in Ghana, Tanzania and Zambia

using ITS based PCR. The detection of new OTUs by the molecular techniques further shows its superiority over commonly used, traditional approaches of diagnosis of coccidiosis since the latter technique could not detect the presence of OTUs.

The inability to detect *E. praecox* and *E. brunetti* by the molecular technique used in the present study could be due to the bias introduced during the harvest of the oocysts (ie caecal harvest at 7 days post infection) which does not favour the chances of getting much of the oocysts of these species. Oocysts of *E. brunetti* are produced in the rectum and are excreted within 7-8 days post infection while *E. praecox* which is found in the small intestine has a prepatent period of 4-5 days post infection (Shirley, 1995). Thus by day 7 post infection most oocysts of *E. brunetti* (which is also a poor oocysts producer) are yet to be matured for subsequent excretion while most of *E. praecox* oocysts are already voided out along with faeces. The number of oocysts of specific *Eimeria* species present in a sample appears to be a limiting factor for its identification by PCR (Haug *et al*., 2007; Kawahara *et al*; 2008; Lee *et al*., 2010; Carvalho *et al*., 2011). The possible presence of faecal contaminants in the harvested oocysts samples may have also affected the sensitivity of the PCR used as contaminants have been reported to affect the quality of extracted DNA and consequently the sensitivity of PCR technique (Carvalho *et al*., 2011). Inadequate disruption of oocysts wall during bead beating could also be a limiting factor for obtaining good quality DNA especially for *Eimeria maxima* that has the toughest oocysts among the chicken *Eimeria* (Haug *et al*., 2007).

In the present study intra- and inter-specific variations in the ITS sequences of the chicken *Eimeria* species was observed and it agrees with previous genetic diversity studies in chicken *Eimeria* (Lew *et al*., 2003; Nowzari *et al*., 2005; Blake *et al*., 2006;

Vrba *et al*., 2011; Swharz *et al*., 2009). However, the present study has gone further, showing that although the genetic diversity among *Eimera* species is widespread it appears not to be dependent on the geographic origin of the isolate as no clear evidence of allopatric selection was observed based on the phylogenetic and principal coordinate analyses of isolates from different geographic and climatic regions. Swharz *et al*. (2009) attempted to associate the occurrence of some particular variants of *Eimeria* species (for example "long" and "short" forms of *E. maxima* and *E. mitis*) to flock performance where they observed two unique *E. maxima* short form variants to be associated with low-performance farms. There is a need for more work to validate the impact of these genetic variation among strains of *Eimeria* species on the performance and management of chicken farms.

The result of the present study has further confirmed the existence of allelic diversity of AMA-1 of *E. tenella*. However, the fact that we observed the occurrence of only one allelic type existing in 10 clones each generated from two different farms, just two allelic types in 10 clones of isolate from another farm and limited diversity amongst globally-collected isolates suggests that the number of allelic types circulating in the population might be very few and may even be translated into fewer AMA-1 isoforms as observed in the present study. Similarly, Blake *et al*. (2011b) reported the widespread occurrence of limited genetic diversity among AMA-1 alleles for *E. maxima* and *E. tenella*. These findings suggest that there may be lower allelic diversity for *Eimeria* AMA-1 than what has been reported from *Plasmodium* parasite, where over 200 unique haplotypes of AMA1 were reported from a single geographical location in Mali for *Plasmodium falcifarum* (Takala *et al*., 2009).

In the present study, it was observed that the nucleotide polymorphism detected in the AMA-1 cDNA resulted in amino acid sequence polymorphisms primarily located in positions 283 -293 and 332 – 386. These polymorphic regions might be associated with the structural development of the binding region of anti-AMA-1 antobodies and therefore explain some degree of strain specific immunity seen in *Eimeria* infection. A hypothesis that needs further confirmation. Amino acid polymorphism has been reported in the binding region of a well-characterized anti-AMA1 monoclonal antibody of the malaria parasite *Plasmodium falcifarum* with resultant mediation of antigenic escape (Dutta *et al*., 2007; Osier *et al*., 2010).

The Houghton strain of *E. tenella* was isolated in the United Kingdom in 1949 and it is one of a small number of defined reference strains used for laboratory studies (Chapman and Shirley, 2003). In the present study we compared the genome of a field strain of *E. tenella* isolated from Nigeria with this archived laboratory strain alongside other laboratory strains (Nippon and Wisconsin strains) and we found that the wild Nigerian strain was more genetically diverse when compared to the H strain than the other two laboratory strains mentioned above. In the wild, cross fertilization between different strains coinfecting a host giving rise to segregation and recombination at the sub-chromosomal level, as well as mutation driven by immune and drug selection, may give rise to novel genotypes, leading to the establishment of genetic variants over time. This might be the reason for the Nigerian isolate being more divergent to the laboratory strains that are kept under controlled condition in the laboratory. Genetic variation among strains of *E. tenella* have been previously reported by other authors (Loo *et al*., 2010; Lim *et al*., 2012) and was suggested to be attributed to the know ability of the parasite to rapidly develop drug resistance. Several genetic markers have been developed for the

characterization of *Eimeria* genomes, some of which were used to map loci that confer resistance to drugs and immune escape from the host (Blake *et al*., 2006; Loo *et al*., 2010; Lim *et al*., 2012). Application of this whole genome mapping strategy may explain the implication of the genetic variation observed within the genome of *E. tenella* strain examined in the present study.

# CHAPTER SIX

# CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Conclusions

From the findings of the present study, it is concluded that:

1. Subclinical *Eimeria* infection is common and wide spread in the commercial poultry farms in the study area especially in flocks raised under deep litter system, birds at the age of 10 weeks or less and flock size of 500 or less.
2. The presence of *Eimeria tenella, E. necatrix, E. maxima, E. acervulina* and *E. mitis* were confirmed for the first time using novel molecular diagnostic tools in commercial poultry farms in the study area. However, the presence of *E. brunetti* and *E. praecox* was not confirmed using the molecular technique, despite their reported presence by the use of traditional parasitological technique.
3. The genetically variant *Eimeria* species (OTU-X, OTU-Y and OTU-Z) were identified for the first time to be circulating in commercial poultry farms in the study area.
4. There appears to be widespread occurrence of inter- and intra-specific genetic diversity in the ITS sequences of *Eimeria* species that infect chickens. Comparison with isolates from different parts of the world indicates the absence of geographic effect on diversity.
5. There is also widespread occurrence of limited genetic diversity among AMA-1 alleles of wild *E. tenella* isolates from within and outside the country, with limited diversity compared to the archived laboratory strains.
6. There is higher genome-wide genetic polymorphism in the wild Nigerian strain of *E. tenella* compared to the reference laboratory strains.

## 6.2 Recommendations

It is therefore, recommended that:

1. For a successful prevention of outbreak of coccidiosis in poultry houses, proper biosecurity measures, good sanitary measures, good litter management and avoidance of all other factors that will favour sporulation and dissemination of oocysts of *Eimeria* species is necessary.
2. Self-diagnosis and treatment of common poultry infections such as coccidiosis, which is commonly practiced, by a good number of the farmers in the study area should be discouraged. This practice encourages drug resistance by *Eimeria* parasite.
3. Further detailed genetic studies (using independent, less variable genetic markers) and biological investigations (life cycle, epidemiology and pathogenesis) of OTU-X, OTU-Y and OTU-Z is recommended to establish their specific status and importance as pathogens in chickens. Future work should evaluate if currently used anticoccidial vaccines and drugs protect chickens against challenged infection with each of the OTUs.
4. Future studies should also attempt to use the sequence polymorphisms unique to some of these genetic variants to design primers that will be used specifically to survey oocysts isolated from litter samples via PCR analysis.
5. Identifying the major allelic types of AMA-1 circulating in the population for development and testing of the efficacy of a multi-allele AMA-1 vaccine against *in vivo* challenge with various strains of *E. tenella* should also be focused in future studies.
6. A whole genome mapping strategy using known makers that will target loci related to drug resistance and leading vaccine candidate genes should be

employed to determine the implication of the genetic variation observed on the genome of *E. tenella* strain examined in the present study. An attempt that may shed more light on the prospect of developing drugs and or sub-unit vaccines against coccidiosis.

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# APPENDICES

**Appendix 1**: Sequences of ITS-1 and ITS-2 of *Eimeria* species isolated from commercial poultry farms in Zaria and environs.

>Ng01_Eac

GGATGCAAAAGTCGTAACACGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCACACATT
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>Ng02_Eac

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CACTGGTGTATATCTCGAAATTTATGGATGGTCCATCATACATTTATTGTGTGATGGGGGGT
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>Ng03_Eac

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**Appendix 2:** Amino acid sequence of ten clones each of three Nigerian field samples of *Eimeria tenella* apical membrane antigene-1 coding gene.

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