Diversity of *Eimeria tenella* apical membrane antigen-1 from chickens in Mpumalanga province and its *in silico* epitope prediction as a vaccine candidate

By

PETRONELLA N. TENZA (212509150)

A dissertation submitted in partial fulfilment of the requirement for the degree of Master of Science in Genetics

School of Life Sciences

College of Agriculture, Engineering and Science
University of KwaZulu-Natal
Westville Campus, Durban
South Africa

Supervisor: Dr Matthew Adeleke

Co-supervisor: Dr Abiodun Fatoba

08 September 2021

PREFACE

The research in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa under the supervision of Dr MA Adeleke and co-supervision of Dr AJ Fatoba.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: [redacted]

Date: 08/09/2021

[redacted]	Dr MA Adeleke	08.09.2021
Supervisor Signature	Name	Date
[redacted]	Dr AJ Fatoba	09-09-2021
Co-supervisor Signature	Name	Date

DECLARATION : PLAGIARISM

I, Petronella Nokukhanya Tenza, declare that:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;

(iii) this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written, but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this thesis is primarily a collection of material, prepared by me, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

_____

Signed: 

Date: 08/09/2021

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest sense of gratitude to my supervisors, Dr MA. Adeleke and Dr AJ. Fatoba, who supported and gave me excellent knowledge throughout my Master's research. Their kindness, understanding, enthusiasm, trusts, motivation, immense knowledge, and provision of an excellent working atmosphere were highly appreciated.

My gratitude extends to Dr VT. Adeleke and Leah Maharaj for their treasured support, which was influential in shaping my experimental methods and critiquing my results. I hope to work with you in the future. I also wish to appreciate all my friends, lab mates, colleagues and research team of Computational Genomics for the cherished time spent together in the lab.

I acknowledge Centre for High Performance Computing, South Africa for the computer programs and facilities that were used in this project.

My greatest appreciation also goes to my family for always supporting and encouraging me, in particular, my mother (Qwabelezi wam), my father (Mkhabela), my sister (Nkululeko) and my brothers (Muzi, Nhlanhla and Senzo) for always being there and providing encouraging words and strength that contributed to the success of this programme.

I dedicate this work to my son Qophelo whom I conceived during this research; this work is for you Ndodana. You were the most excellent motivator. I love you with all my heart.

And to My God, Shembe, Inkosi Unyazi Lwezulu who made it all possible.

The research was financially supported by Moses Kotane Institute.

TABLE OF CONTENTS

LIST OF TABLES

Page

CHAPTER THREE

CHAPTER FOUR

LIST OF FIGURES

CHAPTER FOUR

Abstract

Coccidiosis has been a significant challenge in the poultry industry. There is a high request for the modification of a cost-effective immunizing agent to curtail this disease. Apical membrane antigen 1 (AMA1) has been reported as a protective antigen in sub-unit vaccine development against several apicomplexan parasites such as *Plasmodium falciparum*, *Eimeria tenella* and *Eimeria maxima*. However, knowledge of genetic diversity in this vaccine candidate is imperative. Also, to minimize the cost and time involved in producing a vaccine, computational vaccine design has received much attention through the immunoinformatics method. Therefore, screening for the potential vaccine epitopes in AMA1 that can induce cellular and humoral immune response through the immunoinformatics technique looks promising. This investigation aimed to detect the level of genetic diversity amid *Eimeria tenella* Apical Membrane Antigen 1(*Et*AMA1) in selected farms in Mpumalanga province and predict vaccine epitopes from this antigen. Four hundred fresh faecal samples were collected from 10 selected broiler chicken farms in Mpumalanga. The samples were screened for *Eimeria* oocyst using a compound microscope, and samples containing oocyst were further screened for *E. tenella* using molecular methods. AMA1 (n=103) was amplified from positive samples for *E. tenella,* and resulted amplicons were sent to Inqaba Biotec for sequencing and analyzed using MEGA6.06 and DnaSP programs. The results revealed low levels of genetic diversity among Mpumalanga *Et*AMA1 sequences which were measured by nucleotide diversity (0.0007) diversity, haplotype diversity (0.113) and haplotype number (3). Correspondingly, the haplotype network revealed 4 haplotypes, 3 of which consist of samples from Mpumalanga. Identification of immunogenic B- and T-cell epitopes from *Et*AMA1 sequences was further carried out and were used to construct a multiepitope vaccine (MEV) using immunoinformatics approaches. The constructed MEV is 311 amino acids long. It was constructed by linking 6 B-cell, 3 $CD8^+$ epitopes and 6 $CD4^+$ epitopes with appropriate adjuvant and linkers. Both adjuvant and linkers were used to increase the immunogenicity of the MEV. The designed MEV was highly antigenic and non-allergenic. The results showed a strong binding affinity of MEV with TLR4. These results suggest that the predicted vaccine could be a significant vaccine candidate against chicken coccidiosis through further experimental validation is still necessary.

CHAPTER ONE

1.0 Introduction

The global human population of 7.6 billion is predicted to increase in the year 2050 and 2100 to 9.8 and 11.2 billion, respectively (Department of Economic and Social Affairs, 2017). Food security is a significant concern due to the increased population size (Fatoba and Adeleke, 2018). In South Africa, the poultry industry remains the most significant contributor to the agricultural sector. Chickens are sources of animal protein for human consumption (SAPA, 2019); however, these animals are susceptible to various diseases caused by *Listeria monocytogenes*, avian influenza, and *Eimeria* species, which decrease their productivity (Yang et al., 2016).

Eimeria species is a ubiquitous protozoan parasite of the phylum Apicomplexa that causes coccidiosis disease in livestock and significantly impacts animal welfare and the agricultural economy (Blake and Tomley, 2014). Coccidiosis is common in the poultry industry and can cause high mortality rates (Chapman et al., 2013).*Eimeria* species have been observed in extensive and intensive farming systems (Lawal et al., 2016). The species of *Eimeria* invade the intestinal mucosa and damage the epithelial cells, consequently leading to inflammation and destruction of the intestinal mucosa. The extensive damage caused by these species leads to both clinical and subclinical signs characterized by inefficient feeding, impaired growth, poor digestion, morbidity, and mortality, which may result in an economic loss (Fornace et al., 2013; Siddiki et al., 2014; Gadelhaq et al., 2015). The highest mortality rates of chickens are observed in communal areas due to low veterinary services, overcrowded pens, and poor ventilation (Baker, 2018).

The control measures against chicken coccidiosis are primarily based on chemoprophylaxis, live or attenuated parasites vaccines, and natural bio-products (Blake and Tomley, 2014; Muthamilselvan et al., 2016). However, the drawbacks of these control measures, such as the increasing drug resistance of *Eimeria* (Lawal et al., 2016) and the high production cost of live vaccines (Shirley et al., 2005; Soutter et al., 2020), have made coccidiosis control difficult. Therefore, there is a demand for alternative vaccines to control this parasitic disease.

In a bid to explore alternative control measures against this disease, studies have reported the potential use of recombinant subunit immunizing agents as a control measure against chicken coccidiosis (Kundu et al., 2017; Lin et al., 2017; Tian et al., 2017). Several protective *E. tenella* antigens have been identified and successfully used as recombinant subunit vaccines.

They include but not limited to Apical Membrane Antigen 1(AMA1), Immune Mapped Protein 1(IMP1) and refractile body protein (SO7) (Blake et al., 2015; Kundu et al., 2017; Rafiqi et al., 2018).

Chickens inoculated with recombinant vaccines containing *Eimeria tenella* Immune Mapped Protein 1 (*Et*IMP1), live *Lactobacillus lactis* anchoring *Eimeria tenella* AMA1 (*Et*AMA1), and *Eimeria tenella* refractile protein (EtSO7) have shown increased levels of interleukin (IL)-4 and IgY sera, Immunoglobulin G (IgG) titres and CD4+ T cells, reduced oocyst yield, augmented body weight gain, and reduced gut lesion respectively (Kundu et al., 2017; Pastor-Fernández et al., 2018; Rafiqi et al., 2018). Over the years, recombinant subunit vaccines have been regarded as highly promising in combating coccidiosis (Pastor-Fernández et al., 2018).

Experimental subunit vaccines have also been delineated for many apicomplexans with variable efficacy levels under pilot or research animal models (Blake et al., 2015). However, the development of recombinant subunit vaccines is a challenge due to different factors such as genetic diversity and the complex life cycle of field parasites (Dutta et al., 2007; Blake et al., 2015). When developing an effective subunit vaccine that will remain useful for an extended period, it is indispensable to realize the impacts of parasite antigenic diverseness and population construction on selecting field populations competent of vaccine escape (Blake et al., 2015).

Studies carried out on *Plasmodium falciparum*, one of the Apicomplexa phylum, revealed varying levels of polymorphism among *P. falciparum* with grounds of clonal and panmictic composition, counting on geographic placement and rates of infection (Annan et al., 2007; Larrañaga et al., 2013). Locus-specific examinations have also perceived considerable diverseness in the *P. falciparum* immunizing agent candidates, such as AMA1 and merozoite subsurface protein 1 (MSP1) (Healer et al., 2004; Simpalipan et al., 2014). However, there is a paucity of information on allelic polymorphism and its potential impact on the efficacy of AMA1 as a vaccine candidate against poultry coccidiosis

Computational based approach such as immunoinformatics is an alternative method used to design cost-effective vaccines based on CD8+ T-cell, CD4+ T-cell and linear B-cell epitopes using different parasite antigens (Michel-todó et al., 2019). The approach has been successfully used to design multiepitope subunit vaccines against diseases, including Toxoplasmosis caused by *Toxoplasma gondii*, a protozoan parasite belonging to Apicomplexa (Foroutan et al., 2018).

Onile et al. (2020) have constructed an immunogenic and non-allergenic multiepitope vaccine against *Toxoplasma gondii* based on the Micronemal protein using several immunoinformatics tools. The vaccine consists of different B-and T- cell epitopes (Onile et al., 2020).

A previous study has shown that vaccines based on macromolecular proteins have been confirmed to be safer, effective, and induce immune responses (both humoral and cellular) (Laddy and Weiner, 2006). Thus, recent research studies on the prevention and treatment of disease caused by Apicomplexa have begun to explore the potential benefits of immunoinformatics approaches in predicting and designing epitope-based vaccines. However, little or no information is currently available on using this novel approach to control *Eimeria* infection. This lack of information leaves a gap that needs to be filled by more research studies. Hence, the current study intends to determine the genetic diversity in *Et*AMA1 and develop cost-effective multiepitope vaccines against avian coccidiosis using the immunoinformatics approach.

1.1 Problem statement

The poultry industry plays a vital role in human livelihood in South Africa by creating job opportunities (Nkukwana, 2019). However, increased poultry birds' production is hindered by many factors, including Mycoplasmosis, Newcastle disease, Aflatoxicosis, and coccidiosis. Coccidiosis is an enteric disease that primarily affects broiler chickens. The emergence of coccidiosis compromises animal well-being and increases mortality rates, resulting in economic loss (Mohammed and Sunday, 2015). Some of the chickens are culled to halt the proliferation of the disease.

The impact of this disease affects poultry production and leads to the loss of jobs of farmworkers. *Et*AMA1 has been classified as one of the essential vaccine candidates for chicken coccidiosis. Studies have indicated diversity among this antigen in *P. falciparum*. However, data on the diversity of AMA1 among *Eimeria* species are scanty. Understanding the regional variations among AMA1 antigens could assist in designing a global vaccine against this disease. Also, the protective role of AMA1 antigen in the control of chicken coccidiosis makes it a promising candidate to explore in the design of alternative vaccines against chicken coccidiosis as the current control measures (drugs and live vaccines) have several limitations and drawbacks. Recent advances in vaccine design through immunoinformatics have received much attention due to its benefits, such as reduced cost and the short time involved in its prediction.

Hence, exploring potential vaccine epitopes that can provide cellular and humoral immune protection against chicken coccidiosis through immunoinformatics techniques is highly essential.

1.2 Research questions

- Is the AMA1 antigen present and genetically diverse in different chickens from the Mpumalanga province?
- Are there potential antigenic and immunogenic epitopes in the global *Et*AMA1 that can be used in epitope-based vaccine design?

1.3 Aim:

- To determine the genetic diversity of *Et*AMA1 from chickens raised on ten different farms across the Mpumalanga province of South Africa.
- To use computational tools and immunoinformatics to design an epitope-based vaccine for coccidiosis.

1.4 Objectives:

- To screen for *E. tenella* species.
- To determine the prevalence of *Et*AMA1.
- To determine the genetic diversity of *Et*AMA1.
- To screen for antigenic, immunogenic, and cytokine-inducing CD8+ T-cell epitopes in *Et*AMA1
- To screen for antigenic, immunogenic, and cytokine-inducing CD4+ T-cell epitopes in *Et*AMA1.

1.5 References

Annan, Z., Durand, P., Ayala, F.J., Arnathau, C., Awono-Ambene, P., Simard, F., Razakandrainibe, F.G., Koella, J.C., Fontenille, D., Renaud, F., 2007. Population genetic structure of Plasmodium falciparum in the two main African vectors, Anopheles gambiae and Anopheles funestus. Proc. Natl. Acad. Sci. U. S. A. 104, 7987–7992. https://doi.org/10.1073/pnas.0702715104

Baker, D.G., 2018. Parasitic diseases, The Common Marmoset in Captivity and Biomedical Research. https://doi.org/10.1016/B978-0-12-811829-0.00017-0

Blake, D.P., Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Jatau, I.D., Ayoade, S., Kawahara, F., Moftah, A., Reid, A.J., Adebambo, A.O., Zapata, R.Á., Rao, A.S.R.S., Thangaraj, K., Banerjee, P.S., Dhinakar-Raj, G., Raman, M., Tomley, F.M., 2015. Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. Proc. Natl. Acad. Sci. U. S. A. 112. https://doi.org/10.1073/pnas.1506468112

Blake, D.P., Tomley, F.M., 2014. Securing poultry production from the ever-present Eimeria challenge. Trends Parasitol. https://doi.org/10.1016/j.pt.2013.10.003

Chapman, H.D., Barta, J.R., Blake, D., Gruber, A., Jenkins, M., Smith, N.C., Suo, X., Tomley, F.M., 2013. A selective review of advances in coccidiosis research, in: Advances in Parasitology. https://doi.org/10.1016/B978-0-12-407705-8.00002-1

Department of Economic and Social Affairs, 2017. World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100 | UN DESA | United Nations Department of Economic and Social Affairs [WWW Document]. United Nations.

Dutta, S., Seung, Y.L., Batchelor, A.H., Lanar, D.E., 2007. Structural basis of antigenic escape of a malaria vaccine candidate. Proc. Natl. Acad. Sci. U. S. A. 104, 12488–12493. https://doi.org/10.1073/pnas.0701464104

Fatoba, A.J., Adeleke, M.A., 2018. Diagnosis and control of chicken coccidiosis: a recent update. J. Parasit. Dis. https://doi.org/10.1007/s12639-018-1048-1

Fornace, K.M., Clark, E.L., Macdonald, S.E., Namangala, B., Karimuribo, E., Awuni, J.A., Thieme, O., Blake, D.P., Rushton, J., 2013. Occurrence of Eimeria species parasites on small-scale commercial chicken farms in africa and indication of economic profitability. PLoS One 8. https://doi.org/10.1371/journal.pone.0084254

Foroutan, M., Zaki, L., Ghaffarifar, F., 2018. Recent progress in microneme-based vaccines development against toxoplasma gondii. Clin. Exp. Vaccine Res. 7, 93–103. https://doi.org/10.7774/cevr.2018.7.2.93

Gadelhaq, S.M., Arafa, W.M., Aboelhadid, S.M., 2015. Molecular characterization of Eimeria species naturally infecting Egyptian baldi chickens. Iran. J. Parasitol. 10, 87–95.

Healer, J., Murphy, V., Hodder, A.N., Masciantonio, R., Gemmill, A.W., Anders, R.F., Cowman, A.F., Batchelor, A., 2004. Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in Plasmodium falciparum. Mol. Microbiol. 52. https://doi.org/10.1111/j.1365-2958.2003.03974.x

Kundu, K., Garg, R., Kumar, S., Mandal, M., Tomley, F.M., Blake, D.P., Banerjee, P.S., 2017. Humoral and cytokine response elicited during immunisation with recombinant

Immune Mapped protein-1 (EtIMP-1) and oocysts of Eimeria tenella. Vet. Parasitol. 244. https://doi.org/10.1016/j.vetpar.2017.07.025

Laddy, D., Weiner, D., 2006. From plasmids to protection: A review of DNA vaccines against infectious diseases. Int. Rev. Immunol. https://doi.org/10.1080/08830180600785827

Larrañaga, N., Mejía, R.E., Hormaza, J.I., Montoya, A., Soto, A., Fontecha, G.A., 2013. Genetic structure of Plasmodium falciparum populations across the Honduras-Nicaragua border. Malar. J. 12, 1–10. https://doi.org/10.1186/1475-2875-12-354

Lawal, J.R., Jajere, S.M., Ibrahim, U.I., Geidam, Y.A., Gulani, I.A., Musa, G., Ibekwe, B.U., 2016. Prevalence of coccidiosis among village and exotic breed of chickens in Maiduguri, Nigeria. Vet. World 9. https://doi.org/10.14202/vetworld.2016.653-659

Lin, R.Q., Lillehoj, H.S., Lee, S.K., Oh, S., Panebra, A., Lillehoj, E.P., 2017. Vaccination with Eimeria tenella elongation factor-1α recombinant protein induces protective immunity against E. tenella and E. maxima infections. Vet. Parasitol. 243. https://doi.org/10.1016/j.vetpar.2017.06.003

Michel-todó, L., Reche, P.A., Bigey, P., Pinazo, M., Gascón, J., Alonso-padilla, J., 2019. In silico Design of an Epitope-Based Vaccine Ensemble for Chagas Disease 10, 1–16. https://doi.org/10.3389/fimmu.2019.02698

Mohammed, B., Sunday, O., 2015. An Overview of the Prevalence of Avian Coccidiosis in Poultry Production and Its Economic Importance in Nigeria. Vet. Res. 3.

Muthamilselvan, T., Kuo, T.F., Wu, Y.C., Yang, W.C., 2016. Herbal remedies for coccidiosis control: A review of plants, compounds, and anticoccidial actions. Evidence-based Complement. Altern. Med. 2016. https://doi.org/10.1155/2016/2657981

Nkukwana, T.T., 2019. Global poultry production: Current impact and future outlook on the South African poultry industry. S. Afr. J. Anim. Sci. 48, 869. https://doi.org/10.4314/sajas.v48i5.7

Onile, O.S., Ojo, G.J., Oyeyemi, B.F., Agbowuro, G.O., Fadahunsi, A.I., 2020. Development of multiepitope subunit protein vaccines against Toxoplasma gondii using an immunoinformatics approach 2, 1–11. https://doi.org/10.1093/nargab/lqaa048

Pastor-Fernández, I., Kim, S., Billington, K., Bumstead, J., Marugán-Hernández, V., Küster, T., Ferguson, D.J.P., Vervelde, L., Blake, D.P., Tomley, F.M., 2018. Development of cross-protective Eimeria-vectored vaccines based on apical membrane antigens. Int. J. Parasitol. 48. https://doi.org/10.1016/j.ijpara.2018.01.003

Rafiqi, S.I., Garg, R., Reena, K.K., Ram, H., Singh, M., Banerjee, P.S., 2018. Immune response and protective efficacy of Eimeria tenella recombinant refractile body protein, EtSO7, in chickens. Vet. Parasitol. 258. https://doi.org/10.1016/j.vetpar.2018.06.013

Shirley, M.W., Smith, A.L., Tomley, F.M., 2005. The biology of avian Eimeria with an emphasis on their control by vaccination. Adv. Parasitol. https://doi.org/10.1016/S0065-308X(05)60005-X

Siddiki, A.Z., Mina, S., Anayet Hasan, M., Touaha Akbar, M., Alam, R., Ashraful Islam, M., Zahan Ira, I., Ayesa, B., 2014. Molecular characterization of Eimeria spp. from chicken by Polymerase Chain Reaction based on species-specific SCAR markers. IOSR J. Agric. Vet. Sci. 7, 13–17. https://doi.org/10.9790/2380-07111317

Simpalipan, P., Pattaradilokrat, S., Siripoon, N., Seugorn, A., Kaewthamasorn, M., Butcher, R.D., Harnyuttanakorn, P., 2014. Diversity and population structure of Plasmodium falciparum in Thailand based on the spatial and temporal haplotype patterns of the C-terminal 19-kDa domain of merozoite surface protein-1. Malar. J. 13, 1–11. https://doi.org/10.1186/1475-2875-13-54

South African Poultry Association, 2019. South African Poultry Association 2019 Industry Profile 94.

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry Coccidiosis: Design and Interpretation of Vaccine Studies. Front. Vet. Sci. https://doi.org/10.3389/fvets.2020.00101

Tian, L., Li, W., Huang, X., Tian, D., Liu, J., Yang, X., Liu, L., Yan, R., Xu, L., Li, X., Song, X., 2017. Protective efficacy of coccidial common antigen glyceraldehyde 3-phosphate dehydrogenase (GAPDH) against challenge with three Eimeria species. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01245

Yang, R., Brice, B., Ryan, U., 2016. Morphological and molecular characterization of Eimeria purpureicephali n. sp. (Apicomplexa: Eimeriidae) in a red-capped parrot (Purpureicephalus spurius, Kuhl, 1820) in Western Australia. Int. J. Parasitol. Parasites Wildl. 5, 34–39. https://doi.org/10.1016/j.ijppaw.2016.01.003

CHAPTER TWO

Literature review

2.0 INTRODUCTION

2.1 Poultry Farming

Poultry farming is a system of animal husbandry where avian (birds), such as turkeys, geese, ducks, and chickens, are housed and reared to produce meats and eggs for consumption. Chickens are either raised for meat (broilers) or for egg-laying (layers), which would also be consumed when they become less productive (Tallentire et al., 2016). Poultry (Chicken) meat and eggs are global nutritional constituents, and the demand for poultry meat and eggs is increasing globally due to the continuous increase in the human population (Narasinaku et al., 2020). In 2016, the global poultry production was about 25 billion (FAOSTAT, 2016), while the current annual global poultry meat and egg production are estimated at 120 million and 1500 billion, respectively (Narasinakuppe et al., 2020).

In 2016, the population of chickens was more than any other bird globally, and the chicken was the most slaughtered land animal in the world (Sanders, 2018). Over 66 billion chickens (Fig. 1a) were consumed all over the world in 2016 (Sanders, 2018) . The number rose to over 68 billion (Fig 1b) in 2018 (Sanders, 2020). The poultry and egg industries are the most substantial agricultural sub-sectors in South Africa. The industry accounted for about R47.9 billion (16.6%) of the gross domestic product (GDP) of South Africa in 2018 (The Poultry site, 2020).

The annual broilers and layers reared in 2016 were higher than that of 2017, and the broilers hatched in 2016 was also higher than that of 2017 (SAPA, 2017). Furthermore, the number of broilers slaughtered in South Africa in 2016 (935.571 million) was higher than 927.147 million butchered in 2017 (SAPA, 2017). The total saleable poultry meat production in South Africa in 2017 was 1.658 million tonnes, of which chicken accounted for 1.657 million tonnes (SAPA, 2017). However, the primary threat to poultry industries is the unprecedented outbreak of diseases (Narasinakuppe et al., 2020) with substantial economic losses.

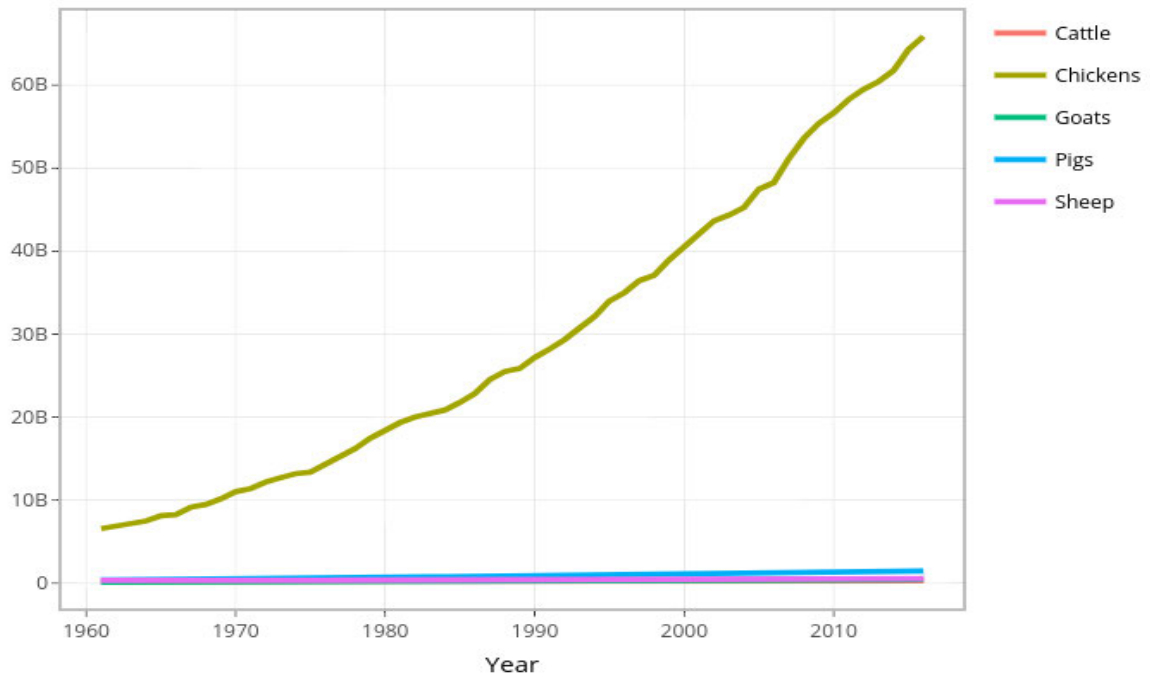

Fig. 1a: Global chickens slaughter statistics and charts from 1961 to 2016.

Source: Global animal slaughter, 1961 – 2016 (Sanders, 2018).

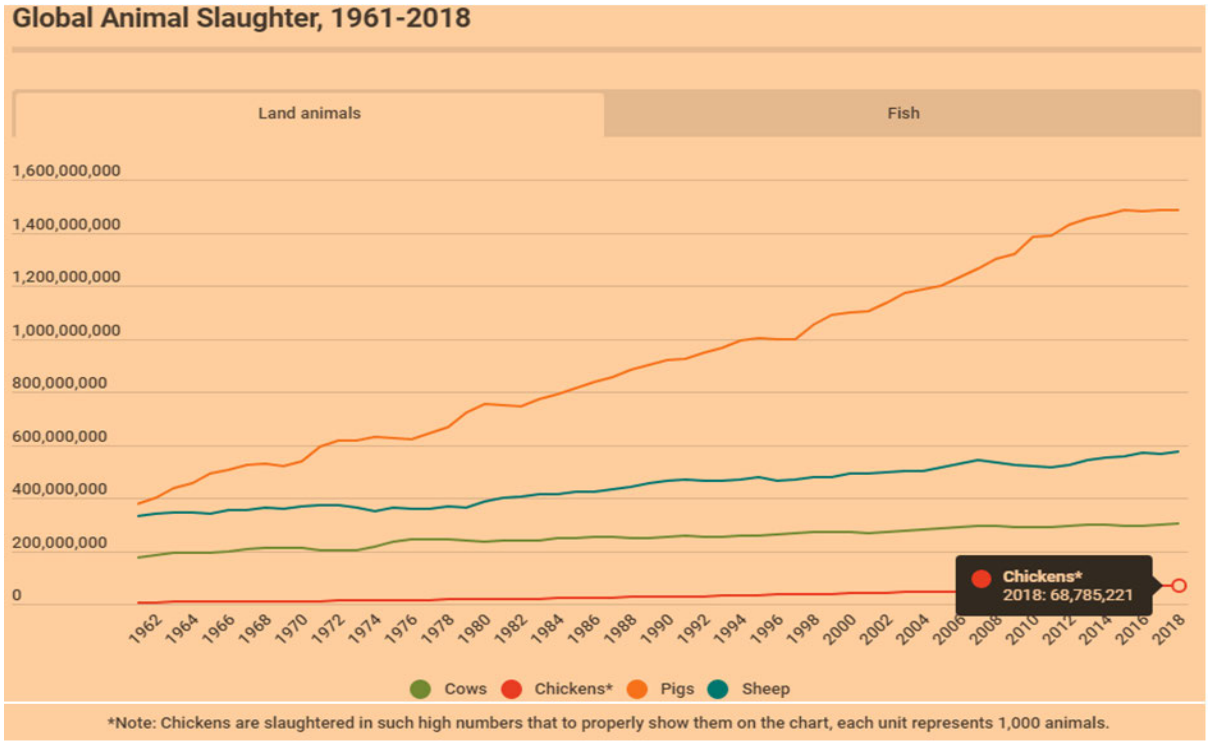

Fig. 1b: Global Animal Slaughter, 1961-2018

Source: Global animal Slaughter, 1961 – 2018 (Sanders, 2020)

2.2 Poultry diseases

The major problem confronting the poultry industry is the outbreak of diseases (SAPA, 2017), which often results in significant economic losses. Poultry diseases are mainly caused by bacteria, fungi, parasites, and viruses. Some of these diseases include mycoplasmosis, necrotic enteritis, new castle disease, and coccidiosis (Moore, 2016; Souillard et al., 2017; Fatoba and Adeleke, 2018; Al-Mamun et al., 2019). It is estimated that the global economic impact of chicken diseases and the cost of treatments and other control interventions might cost the chicken production industry to the tone of £2 billion annually (Dalloul and Lillehoj, 2006; Soutter et al., 2020).

2.2.1 Coccidiosis

Chicken coccidiosis is a gastrointestinal disease caused by the rapid build-up of intracellular apicomplexan parasites (Allen and Fetterer, 2002). Coccidiosis is one of the most frequent enteric illnesses affecting broiler chickens (Borgonovo et al., 2020), which leads to reduced performance, reduced welfare, increased mortality, and a higher risk of product contamination (Abdelli et al., 2020). Coccidiosis may deter maturation and feed conversion of contaminated animals' leading to the consequent loss of productiveness (Dalloul and Lillehoj, 2006). The global yearly costs imposed by coccidiosis to commercialized domestic fowl have been approximated at £2 billion yearly, accentuating the demand for expeditious plans of action to control this organism (Peek and Landman, 2011). It has been noted that environmental variables, such as the high density and the vast number of broiler chickens reared per time, might encourage coccidiosis development (McDougald et al., 2019).

2.2.1.1 Etiology of the chicken coccidiosis

Coccidiosis is an illness originated by parasites of the taxonomic category *Eimeria* from the phylum Apicomplexa (Soutter et al., 2020). Though most species are strictly host-specific, *Eimeria* can cause coccidiosis in all livestock (Kvičerová and Hypša, 2013). Chicken coccidiosis is caused by at least seven species of *Eimeria* namely *Eimeria acervulina, E. mitis, E. tenella, E. maxima, E. brunetti, E. necatrix, and E. praecox* (Chapman, 2014). Each species varies in its fatality, pathogenicity, and site of infection within the host's gastrointestinal tract (Chapman, 2014; Soutter et al., 2020). Furthermore, infection with each strain of *Eimeria* elicits an immune response in reaction to homologous exposure (Rose, 1963).

Notwithstanding, the numbers of parasites and rounds of transmission require to develop an adequate immunity to defend in reaction to malady vary counting on the infecting *Eimeria* species, dosing agenda, and chicken lineage (Soutter et al., 2020).

Eimeria species also alters between geographic location and poultry industries and may be a determinant of parasite genetic diverseness (Chengat et al., 2017). The invasion and colonization of the host's gastrointestinal tract by *Eimeria* species generally amend the intestinal epithelial cells, tissues of the caecum and interrupts gut homeostatic balance (Chen et al., 2020). Furthermore, *Eimeria* infection increase and predispose chicken to gut colonization and co-infection by pathogenic microorganisms such as *Gallibacterium anatis* (Narasinakuppe et al., 2020), *Clostridium perfringens* (Stanley et al., 2014) and also lead to shifts in microbial community structure (Stanley et al., 2014).

2.3 *Eimeria tenella*

Eimeria tenella is the most infective and one of the most frequent isolated *Eimeria* species implicated in chicken's coccidiosis (Blake et al., 2015). *Eimeria tenella* infection is localized only in the caecum and characterized by the presence and accumulation of blood in the caecum and by bloody droppings. Also, tissue debris, accumulations of thrombus and oocysts, may be detected in chickens that survived the benign stage of the infection (Gerhold, 2014) *E. tenella* is the model immunogenic strain generally used for the biological and immunological characteristics of the apicomplexans. It is considered the vaccine delivery vehicle capable of expressing pathogen antigens (Tang et al., 2018b). It elicits acquired antigen-specific immune responses in chickens and other mammals (Marugan-Hernandez et al., 2016). *Eimeria tenella* generally infects epithelial cell of the caecum crypts of Lieberkuhn, eliciting different ranges of pro-and anti-inflammatory cytokines such as interleukin (IL)-6, IL-17A, IL-10 and interferon (IFN)-γ (Macdonald et al., 2017). Transmission may also lead to haemorrhagic lesions of varying degree, influenced by parasite oocyst dose magnitude and period of life of the bird, as well as host genetic constitution and former infection chronicle (Johnson and Reid, 1970; Boulton et al., 2018).

2.3.1 The life cycle of *Eimeria tenella* species

The life cycle of the *E. tenella* is the same for all *Eimeria* species and is categorized into three (sporulation, schizogony, and gametogony) stages: (i) formation of infectious and sporulated oocyst (sporulation), (ii) asexual reproduction resulting in the multiplication of the etiological agent in the intestine of the host (schizogony), and (iii) sexual reproduction leading to the formation of gametes (gametogony), which becomes unsporulated after fertilization (Chapman and Jeffers, 2014). A typical life cycle of *Eimeria spp* in chicken (Fig. 2) begins with the consumption of matured (sporulated) oocysts (after excystation in the gastrointestinal tract of the host) to form the sporozoites (Belete et al., 2016), which would, in turn, invade host cells in preparation for asexual reproduction (schizogony). One sporulated (infective) oocyst is formed by four sporocysts, and one sporocyst contains two sporozoites; therefore, each oocyst releases eight sporozoites after excystation (Ahmad et al., 2016). The release of the sporozoites from the oocyst is facilitated by bile salts and chymotrypsin (Jenkins et al., 2019).

After excystation in the duodenum of the host, the sporozoites invade the host's enterocyte and encapsulate within the parasitophorous vacuole (while protecting itself from the host's phagolysosomes) and begin asexual reproduction (schizogony or merogony) (Tabarés et al., 2004; Jiang et al., 2012). During invasion and encapsulation processes, secretory organelles (rhoptries, micronemes and concentrated granules) release proteins that assist penetration and infestation (Jiang et al., 2012). After several asexual cycles, merozoites are then released from the schizonts (to begin re-infection, they rupture the host cell), and the schizonts invade new ones (infective cycle).

After several merogonic divisions, the merozoites undergo gametogony (to produce male and female gametocytes after invading the enterocytes) and begin sexual reproduction (Ahmad et al., 2016). The stage ends with the production and release of oocysts into the intestinal lumen of the host. After fertilization, the unsporulated oocysts are released and ejected into the environment with the host's faeces (Ahmad et al., 2016). Once in the environs, the oocysts must experience sporulation to be able to infect another host (Shapiro et al., 2019). Furthermore, all *Eimeria* species require only one host throughout their faecal-oral lifecycles, given room for the control of their replication in vivo and transmission in the environment (Blake et al., 2017).

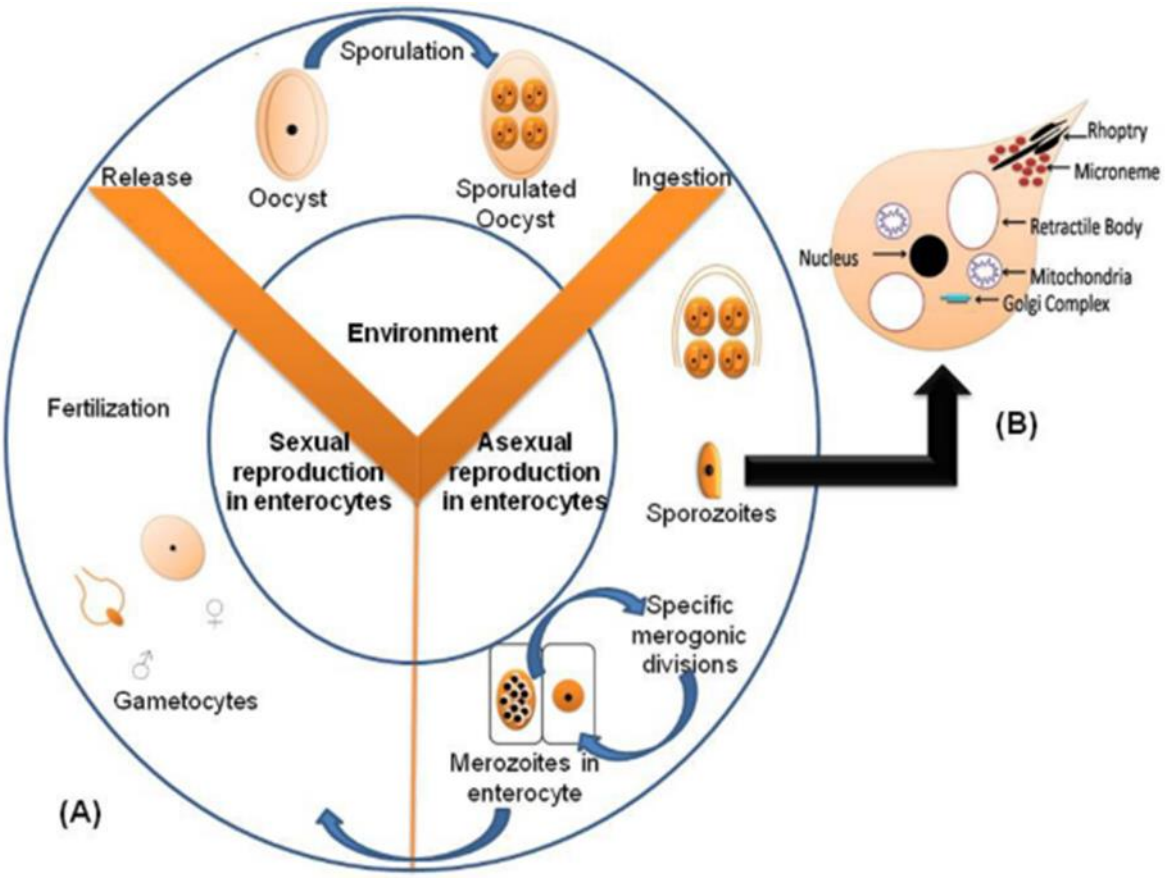

Fig. 2. A typical life cycle of Eimeria spp.

Source: Ahmad et al., 2016 (Ahmad et al., 2016)

2.4 Traditional and Molecular identification and diagnosis

Detection and genetic delineation of *Eimeria* are crucial to hindrance, surveillance, and control of coccidiosis (Morris and Gasser, 2006). Identifications of *Eimeria* species involve traditional and molecular approaches. The traditional methods involve the characterization of each *Eimeria* species based on oocyst morphology and pathologic observation of the abscessed intestine (Johnson and Reid, 1970). This includes clinical characteristics such as lesions in specific intestine sites, structural and biological attributes such as the magnitude of oocysts, sites of contagion, pre-patent period, and sporulation instance. Among *Eimeria* species, *E. maxima* are well known to be founded on oocyst magnitude, while *E. tenella* and *E. necatrix* create apparent lesions (Mohammad et al., 2011). Detection through these parametric qualities is subjectively ascribable to overlapping characteristics (Long et al., 1982). Additionally, it is presumptive, pricey, time-consuming (Haug et al., 2008) and requires highly trained personnel (Hinsu et al., 2018).

The challenges mentioned above have been mainly overcome via the molecular approach, which includes polymerase chain reaction (PCR) (Haug et al., 2008), quantitative polymerase chain reaction (qPCR) and loop-mediated isothermal amplification (LAMP) (Barkway et al., 2015). The PCR, qPCR and LAMP approaches are constricted by fluctuation in target succession diverseness and strenuous guide preparation (Hinsu et al., 2018). Polymerase chain reaction-based method established to be useful for the determination of the seven *Eimeria* species in poultry. The commonly used target regions are small subunit rRNA (Tsuji et al., 1997), 5S rRNA (Stucki et al., 1993) internal transcribed spacers 1 and 2 (ITS 1; ITS 2) of nuclear ribosomal DNA (Haug et al., 2008), sequence-characterized amplified region (SCAR) derived from random amplified polymorphic DNA (RAPD) profiles (Fernandez et al., 2004) and quantitative PCR (qPCR) (Kundu et al., 2020). The presence of isothermal enzymes and intercalating dyes LAMP signified that the reaction could be run in the absence of a thermocycler and electrophoresis, which might make the strategy to be more advantageous than the molecular techniques if the method is optimized and approved as one of the standard protocols (Fatoba and Adeleke, 2018).

Eimeria species genomes are commonly sequenced using 18S rDNA and ITS 1 and 2 sequences (Schwarz et al., 2009). The ITS sequences are frequently used for molecular diagnostics and phylogenetic typing based on the uniqueness rendered by their advanced copy number and comparatively rapid organic evolution (Schwarz et al., 2009). The method is, however, limited by intra-clonal polymorphism (Schwarz et al., 2009). Moreover, genotypic analysis targeting ITS 1 and 2, 18S rDNA, and mitochondrial cytochrome C oxidase subunit I (mtCOI) has been used as molecular diagnostics of different *Eimeria* species (Kumar et al., 2014; Hafeez et al., 2015). Although mtCOI has been suggested as the best phylogenetic marker, 18S rDNA is currently the most commonly sequenced *Eimeria* gene with the most extensive public dataset of reference sequences (Miska et al., 2010).

Hinsu *et al*., 2018 explored Illumina MiSeq deep sequencing's fidelity to identify and quantify *Eimeria* populations in commercialized broilers and indigenous poultry and compare the sensitivity of MiSeq deep sequencing analysis with those of ITS (Hinsu et al., 2018). The authors found that the SCAR numeric PCR assays indicated higher sensitivity of the MiSeq strategy. The emergence of qPCR and its alternatives, such as the Sequenom assay single nucleotide polymorphism (SNP) panel (Blake et al., 2015), have allowed the characterization and quantification of the seven known *Eimeria* species of poultry (Kundu et al., 2020). The qPCR is more superior to the conventional PCR and normalized protein catabolic rate (nPCR).

It involves single-step amplification and quantification. As such, there is no need for post-amplification electrophoresis and visualization of PCR products, thereby reducing time and minimize chances of contamination during PCR (Chengat et al., 2017; Kundu et al., 2020). However, the cost of acquiring real-time thermal cycler equipment and reagents is expensive (Kundu et al., 2020).

2.5 Control of *Eimeria* coccidiosis

Coccidiosis control in chicken production depends on routine chemoprophylaxis and vaccination with live virulent or attenuated *Eimeria* species (Soutter et al., 2020). The chemoprophylaxis is primarily used for broiler chickens. In contrast, layer and breeder chickens are usually vaccinated with live virulent or attenuated *Eimeria* parasites. About 70% of anticoccidial drugs used to date are ionophores (Eckford et al., 2013). Ionophores provide partial anticoccidial protection even against naïve field isolates at concentrations that are not cyanogenic to chickens (Blake et al., 2017). This allows the etiological agents to continue duplicating while Ionophores are being administered (Chapman, 1999).

Ionophores facilitate the contact of chickens with actively replicating *Eimeria* that confers natural immunity that protects chickens upon withdrawal of medications before the onset of egg production of slaughtering (Chapman, 1999). The efficacy of ionophores has been primarily attributed to their leakiness (Blake et al., 2017). However, low-level parasite diversion from ionophore medications has been a prevailing pitfall (Bafundo et al., 2008). Incomplete eradication of the etiological agents has slowed down the selective pressure in the direction of drug resistivity, which possibly has prolonged the transactional value of ionophores (Blake et al., 2017).More than 40% of poultry farmers (in the United States and other countries) are now considering immunoprophylaxis for their chickens due to the reclassification of ionophores as an antibiotic and quest for antibiotic-free food (Peek and Landman, 2011; Chapman and Jeffers, 2014).

Also, the resistance of *Eimeria* to every anticoccidial drug presently available often occurs within one year of release (Chapman, 1997; Blake et al., 2017). This quick response of *Eimeria* species to resist the potent effect of lethal anticoccidial drugs indicated advanced cognition for genome plasticity and variegation. It underlined the presence of pre-existing genetic multifariousness within *Eimeria* genomes (Blake et al., 2017).

Anticoccidial vaccines prepared using live *Eimeria* species are an efficient alternative to chemoprophylaxis as the ingestion and recycling of controlled doses of vaccine oocysts elicits robust immune protection (Soutter et al., 2020). The first generation anticoccidial vaccines were prepared using live, wild type, and sporulated *E. tenella* oocysts and were introduced in the early 1950s based on the fact that administration of low doses of oocysts for days elicits healthy protective immunity against homologous challenge (Williams and Gobbi, 2002; Blake et al., 2017). These first sets of anticoccidial immunizing agents were formulated to integrate other *Eimeria* species and have been widely explored (Williams and Gobbi, 2002).

Vaccination of chicken with oocysts is not always uniform, and employment of live oocysts by contagion is requisite for the proper improvement of adequate immune protection within the chickens (Price et al., 2014). Although the use of floor covering has been demonstrated to enhance oocysts recycling, the process is problematic in traditional layer cage setups (Price et al., 2016). The major drawback in the utilization of wild-type, highly infective, or live vaccines is that a significant number of oocysts amass within the poultry farm, which may often lead to a high level of exposure and disease with mortality in some cases (Williams and Gobbi, 2002).

The second-generation anticoccidial immunizing agents are developed from oocysts obtained from weakened *Eimeria* species. Most of them have been selected for precocious development as reviewed by Shirley & Bedrnik, 1997 (Shirley and Bedrník, 1997) . Precocious parasite lines are characterized by reduced pathogenicity but significantly sustained immunogenicity (McDonald and Ballingall, 1983). A model and potent precocious cell is the *E. tenella* line designated for maturation in embryonic young birds (Long et al., 1982). The attenuated vaccine is made up of an intermixture of sporulated oocysts from various *Eimeria* species, and it can be administered orally, akin to those of live wild-type immunizing agent (Soutter et al., 2020). Live anticoccidial immunizing agents have been grossly utilized in domestic fowl commercial enterprise for over 50 years with no grounds of parasite development toward resistance/immune diversion (Soutter et al., 2020). Naturally present genetic diverseness has been dependably defeated by the involvement of more than a single "strain" of a single taxonomic group in some vaccine amalgamations (Shirley et al., 2005). These live vaccines have to be grown in poultry, and as such, contributing to the cost of production of this vaccine (Shirley et al., 2005; Soutter et al., 2020). Manufacture of live wild-type and the attenuated anticoccidial immunizing agent is also time-consuming (compared to anticoccidial drugs) and are constricted by the need for in vivo infection of the hosts (chickens) to produce infectious oocysts which cannot be obtained in vitro (Soutter et al., 2020).

Regrettably, existent vaccines are comparatively costly due to advanced industry costs and are challenging to scale up, particularly those containing weakened parasite lines (Soutter et al., 2020). In recent times, the demand for cost-effective anticoccidial vaccines is more significant than ever (Soutter et al., 2020). Anticoccidial vaccine usage in the poultry has also been restricted by the necessity for multiple immunizing agent lines of parasites produced by a self-directed transition in chickens (Soutter et al., 2020).

2.6 Population structure and genetic diversity of *Eimeria tenella*

In-depth knowledge of the population constitution of *Eimeria* species is paramount for understanding the epidemiology of coccidiosis and for developing effective control measures (Blake et al., 2015). Each *Eimeria* species' regional occurrence and the degree of vaccine-targeted antigenic diverseness evolve greater importance as recombinant or vectored subunit anticoccidial immunizing agents have become more available (Blake et al., 2011; Blake and Tomley, 2014). Determining the level of genetic diverseness across parasite genomes is very applicable to understanding pathogen continuity and development (Blake et al., 2017). The pre-existing genetic diversity across pathogens is often influenced by a change in selective pressure and the rate of adaptation for pathogen survival (Blake et al., 2017). The extent of intra-specific multifariousness has been studied in few *Eimeria*, but appreciable genetic fluctuation has been identified between taxonomic groups that have been studied (Blake et al., 2017).

Genome-wide genetic multifariousness among *E. tenella* strains has been studied using different approaches. Blake *et al*., 2004 and Reid *et al*. 2014 separately evaluated genetic polymorphism amid the *E. tenella* (Houghton, United Kingdom) reference strain and a panel of archive *E. tenella* strains using amplified fragment-length polymorphism (AFLP) to determine Jaccard indices of the variety of *E. tenella* in the deficiency of more all-embracing genomic resources (Blake et al., 2004; Reid et al., 2014). Comparative analysis of *E. tenella* (Houghton, United Kingdom) strain with the LCH2 and Weybridge (United Kingdom.), Beltsville and Wisconsin (United States), and Nippon-2 (Nt2; Japan) estimated Jaccard indices of 0.912, 0.967, 0.956, 0.914, and 0.873, respectively (Blake et al., 2015).Clark *et al*., 2016 studied the comparative analysis of *Eimeria* species using fixation indices of 248 *Eimeria* ITS sequences 1 and 2 (comprising *E. tenella, E. acervulina* and *E. mitis*, respectively) and found significant intra-specific diversity, with proof of possible allopatric variation only in *E. tenella* sequences but not in other *Eimeria* species analysed (Clark et al., 2016).

The genetic diversity of genotypically confirmed *E. tenella* strains from Northern India (n =86), Southern India (n = 53), Egypt (n = 40), Libya (n = 51), Egypt and Libya (n = 91) and Nigeria (n = 14), were evaluated (Blake et al., 2015), using a genome-wide panel of fifty-two informative single nucleotide polymorphisms (SNPs) (Blake et al., 2015). The analysis identified ninety-three distinct *E. tenella* haplotypes from the 244 *E. tenella* strains. However, linkage disequilibrium (LD) studies show apparent differences between geographical locations (Blake et al., 2015, 2017).

Investigation of the incidences of haplotypes and diversity showed an increase of distinct haplotypes in Northern Africa (Libya and Egypt) and Northern India with significant LD, respectively, contrary to those found in Nigeria and Southern India (Blake et al., 2015). The available haplotype diversity detected in Northern Africa, and Northern India is partially similar to that of coccidian *Toxoplasma gondii,* in which a smaller number of governing clonal genotypes have been reported, coupled with the higher level of genetic diversity in Southern America (Su et al., 2012). Nevertheless, comparison between these two genera uncovers rudimentary biological divergence (Blake et al., 2015).

In Nigeria and Southern India, haplotypes were only detected once, which indicated that sexual union and recombination happen at large in these populations, and only a few genetic varieties may have been observed (Blake et al., 2015). This type of cryptic sexuality and genetic diversity indicates advanced levels of parasite transmittance; however, clonal extermination is also plausible (Ramírez et al., 2012). The genetic variation in *E. tenella* may also be due to the presence and increased levels of poly-haplotype contagion in the regions with more haplotype complexity which may favour hybridization (Blake et al., 2015). The lower parasite presence in Northern India and North Africa would be anticipated to cut back on opportunities for cross-fertilization, consequent in a more restricted enlargement of a smaller number of haplotypes with or without proper clonal improvement (Blake et al., 2015). The levels of genetic diversity were conspicuous in *E. tenella* populations from India, where the strains from Northern India chickens indicated a restricted diversity in which seven out of the eight *E. tenella* haplotypes detected among the eighty-six chickens were unique to the region, unlike Southern India strains, where forty-nine out of the fifty *E. tenella* haplotypes detected were unique to that particular geographical location (Blake et al., 2017). Also, comparative analysis of *E. tenella* from different geographical locations indicated that that 98% and 87.5% of the identified *E. tenella* haplotypes were incomparable to their specific regions, buttressing the existence of allopatric variegation (Blake et al., 2015).

However, Egypt and Libya *E. tenella* strains indicated a mixed haplotype character, which also shows a similar regional specificity level (Blake et al., 2015). The efficacies of the current anticoccidial immunizing agents against genetically different *E. tenella* strains were also elucidated (Blake et al., 2015). The researcher observed that the observed genomic diversity among *E. tenella* is connected with a narrow escape from strain-specific immune termination. Although *Eimeria* species that pollute chickens are ubiquitous, *E. tenella* displays uncertain population artefact with a resultant fluctuation in the probability of hereditary conversation (Blake et al., 2015).

2.7 Anticoccidial vaccine candidates associated with *Eimeria tenella*

Eimeria species antigenic agents are classified as possible immunizing agent prospects founded on their host-parasite penetration, fundamental action, and multiplication (Suprihati and Yunus, 2018). Novel immunoprotective *Eimeria* antigens have been characterized using different strategies (Kundu et al., 2017; Rafiqi et al., 2019; Zhang et al., 2020). However, detection and characterization of immune protective genes loci using expression library immunization seem useful for new vaccine development (Arafat and Abbas, 2018). Most of these antigens are produced in the micronemes of the parasite to enhance attachment, the commencement of mobility and the outlet from the host (Liu et al., 2017, 2018).

The antigenic proteins that have been characterized from *Eimeria tenella* to date include AMA1, elongation factor (EF-1/2), ubiquitin-conjugating enzyme (UCE), recombinant refractile body protein (EtSO7), profilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glycosylphosphatidylinositol (GPI)-linked surface antigens (TA4), microneme protein (MIC1, 2 and 3) and Gam22 (Allen and Fetterer, 2002; Lai et al., 2011; Liu et al., 2017; Rafiqi et al., 2018; Yan et al., 2018). Apical membrane antigens (AMAs) are a unit of integral membrane protein present in obligate intracellular apicomplexan parasites (for instance, coccidian *Eimeria* species, *Toxoplasma gondii*, *Plasmodium* species etc.) and are considered as a novel vaccine candidate (Zhang et al., 2015; Pastor-Fernández et al., 2018). AMAs are crucial for the placement and repair of adhesion between parasites and hosts cells membranes (moving junction), through which the parasites move (glide) into the intracellular vacuole (Tyler et al., 2011; Pastor-Fernández et al., 2018).

Phylogenetic and genomic analysis have classified four AMA paralogues (AMA1-4) that are unique to coccidian and haemosporine apicomplexans, including *Eimeria* species (Parker et al., 2016; Pastor-Fernández et al., 2018). Nonetheless, proteomic investigation of *E. tenella*

recognized two dominant stages ordered by AMAs (*Et*AMA1 and *Et*AMA2) that are expressed explicitly by *E. tenella* sporozoites (*Et*AMA1) and merozoites (*Et*AMA2) (Oakes et al., 2013; Pastor-Fernández et al., 2018). Also, Blake *et al*., 2015 studied the genetic variance in *E. tenella* and found little inherent polymorphism at the AMA1 gene locus (Blake et al., 2015).

Pastor-Fernández *et al*., 2018 found that both antigens are stage-regulated, with *Et*AMA1 being sporozoite-specific, while *Et*AMA2 is merozoite specific. The sporozoic-specific *Et*AMA1 effectively elicits partial immune security against a homologous state of affairs with *E. tenella* when used as a recombinant protein immunizing agent. In contrast, the merozoite-specific *Et*AMA2 did not elicit an immune response (Pastor-Fernández et al., 2018).

Li *et al*., 2018 evaluated the cautionary effects of live *Lactobacillus lactis* anchoring *Et*AMA1 protein against *E. tenella* challenge founded on oocyst decrease ratio, bodyweight increase, and lesion score in the caeca. The reports showed that the chickens vaccinated with the transgenic live *L. lactis* expressing *Et*AMA1 protein provided more immune protection (characterized by the presence of higher IgG titres and CD4+ T cells proportions) against homologous challenge, suggesting that *Et*AMA1 protein expressed by a recombinant live *L. lactis* clone could induce effective immune responses against *Eimeria* infection (Li et al., 2018). Micronemes organelle proteins (MICs) are tiny membrane-bound organelles (situated below the cell membrane, close to the antecedent end of the apical complex) that are composed of mixtures of soluble and transmembrane proteins (Tomley et al., 1991; Periz et al., 2007). They also contain multiple copies of adhesive protein domains (Periz et al., 2007). MICs are expressed in the early period of host cell intrusion, and they aid in the recognition and attachment of parasites to the host cells and forming of actinomyosin system, which enhances the invasion (Huang et al., 2018).

Eimeria tenella MICs form a new and ultra-high molecular mass protein complex that binds target host cells (Periz et al., 2007). *Eimeria* species invades the host intestinal cells with the aid of glideosomes and the secretion of adhesive proteins from the micronemes onto the host surface (Periz et al., 2007). *Eimeria tenella* microneme 1 protein (*Et*MIC1) is the first microneme protein identified in *E. tenella* (Tomley et al., 1991). *Et*MIC1 is a transmembrane protein that could play a prominent role in developing the *E. tenella* parasitophorous vacuole (PV) (Zhao et al., 2019). In vitro analysis has shown that *Et*MIC1 could be secreted into the culture medium during host cell invasion (Bumstead and Tomley, 2000) and could interact with *Et*MIC2 (Rabenau et al., 2001). Furthermore, *Et*MIC1 is expressed in all developmental stages of *E. tenella* (Liu et al., 2014).

Two epitopes of *Et*MIC1 have also been detected and labelled at different developmental stages of *E. tenella* using monoclonal (mAbs; 1-A1 and 1-H2) antibodies (Liu et al., 2014; Zhao et al., 2019).

*Et*MIC2 is an acidic microneme protein that was first detected in 1996 (Tomley et al., 1996). Since its detection, several studies (Wang et al., 2014; Zhang et al., 2014; Yan et al., 2018) have alluded to its high immunogenic potential and suggested that *Et*MIC2 might be a good candidate for a novel DNA vaccine. The *Et*MIC2 gene encoding a 35.07 kDa *Et*MIC2 protein in a sporulated oocysts of *E. tenella* had been cloned and recombinant (r*Et*MIC2) protein expressed in a competent *E. coli* to determine its potential as a novel DNA vaccine candidate (Yan et al., 2018). *Et*MIC2 protein was confined mainly in the cytoplasm of first-and second-generation merozoites, membrane and anterior region of *E. tenella* sporozoites and was expressed during the first stage of schizogony (Yan et al., 2018).

Additionally, incubation with particular antibodies against *Et*MIC2 was established to lessen the ability of *E. tenella* sporozoites to enter host cells. Immunolocalization studies with recombinant *Et*MIC2 protein (pcDNA3.1(+)-*Et*MIC2) importantly enhance weight increase while lesion score and oocyst production in chicken were significantly reduced (Yan et al., 2018). The study has also suggested that *Et*MIC2 plays a critical role in the host cell intrusion and may be an executable candidate for developing brand-new immunizing agents against *E. tenella* infection in poultry (Yan et al., 2018). Tomley *et al*., 1996 also showed that *Et*MIC2 forms a complex with *Et*MIC1, and the complex (MIC1-MIC2) presumptively became mobilized from the micronemes to *E. tenella* surface during attachment and redistributed towards the posterior end of the parasite during the incursion of the host cell (Tomley et al., 1996).

Eimeria tenella microneme 3 (*Et*MIC3) protein is a 130 kDa soluble microneme protein with eight cysteine-rich repeats of 108 and 151 residues (Lai et al., 2011). *Et*MIC3 has five highly similar microneme adhesive repeat (MARs) (Labbé et al., 2006; Blumenschein et al., 2007; Lai et al., 2009), which are 14 kDa protein domains localized entirely in apicomplexan parasites (Lai et al., 2009). The second MAR domain was confirmed to be a receptor for sialyl oligosaccharides and termed MAR1a (*Et*MIC3-MAR1a) using complete resonance assignments (Lai et al., 2011). *Et*MIC3 is expressed in the micronemes of merozoites and sporozoites from a single-copy gene and is secreted onto the *E. tenella* surface during host cell invasion (Lai et al., 2009).

*Et*MIC3 play a prominent role in host cell intrusion and specificity (Lai et al., 2011; Zhang et al., 2020). Recombinant *Et*MIC3 (pVAX-EtMIC3) protein elicits a robust immune response characterized by momentous lymphocyte proliferation, cytokine induction and antibody outcome in chickens upon vaccination (Wang et al., 2017).

Eimeria tenella microneme 4 proteins (*Et*MIC4) gene encode a 240 kDa transmembrane protein which incorporates thirty-one tandemly arranged calcium-binding epidermal growth factor (EGF)-like repeats (Periz et al., 2005; Zhou et al., 2012) and sixteen thrombospondin type I within its extracellular domain (Periz et al., 2007). The tandemly arranged calcium-binding epidermal growth factor repeats within *Et*MIC4 supply a scheme whereby, in the calcium-rich extracellular surroundings, the protein could assume a protease-resistant, rigid structure that could vantage its interaction with host cell ligands (Periz et al., 2005). *Et*MIC4 also shares advanced sequence homology with the *E. maxima* protein EmTFP250 and *Toxoplasma gondii* microneme protein (TgMIC12) (Periz et al., 2009).

Eimeria tenella microneme 5 proteins (*Et*MIC5) gene expressed a 100 kDa soluble *Et*MIC5 protein that contains eleven domains belonging to the plasminogen-apple-nematode (PAN) superfamily (Brown et al., 2003). *Et*MIC5 also contains 11 tandemly recurrent domains belonging to the plasminogen-apple-nematode superfamily (Periz et al., 2007). *Et*MIC3 and *Et*MIC5 antigens bind to the sialic acid molecules within the epithelial cell, promoting host cell invasion (Pastor-Fernández et al., 2018). Both *Et*MIC4 and *Et*MIC5 interact to shape an oligomeric, ultrahigh molecular mass protein complex within the host secretory pathway and is conserved on the hosts' surface after secretion (Periz et al., 2007).

Profilin (170 amino acids open reading frame) is a preserved surface antigen of merozoites and sporozoites of *E. tenella* expressed during invasive, sporozoitic and merozoitic phase of *E. tenella* life cycle (Zhang et al., 2012; Lillehoj et al., 2017). According to Tang *et al*., 2018, chicken immunized with transgenic *Eimeria tenella* (*Et*EmPro) expressing the profilin of *E. maxima* elicited higher antigen-specific cell-mediated immunity, with increased concentrations of interferon gamma-secreting lymphocytes (Tang et al., 2018b). The recombinant *Et*EmPro also provide greater immune protection against *E. tenella* challenge than the wild type (Tang et al., 2018b). Also, the heterogeneity of the faecal biota of chickens vaccinated with *Et*EmPro differed from that of the wild-type-immunized chickens' microbiome, which shows the interactions of *E. tenella* with the gut microbiome of chickens (Tang et al., 2018a).

The report also showed that ginsomes adjuvant in complex with recombinant *E. tenella* profilin antigen could effectively induce a subunit immunizing agent to elicit a robust immune response and cautionary effects (Zhang et al., 2012).

The immune response and efficacy of genetically engineered *Lactobacillus lactis* strain (NZ9000) harbouring *E. tenella* profilin 3-1E protein and dendritic cell-targeting peptide (DCpep) against *E. tenella* sporulated oocyst was also evaluated (Li et al., 2020). Chickens orally immunized with the transgenic *L. lactis* (NZ9000-DCpep-3-1E) clone showed a higher immune response (against *E. tenella* sporulated oocyst), characterized by (i) high levels of profilin 3-1E specific serum IgG and secretory IgA (sIgA) in caeca lavage, (ii) high levels of CD4+ and CD8α+ cells in peripheral blood, and (iii) higher mRNA expression levels of IL-2 and IFN-γ in chickens spleen compared to *L. lactis* expressing profilin 3-1E (Li et al., 2020).

Refractile body protein (SO7) is an essential immunogenic protein involved in the early stages of infection development of *Eimeria* species (Rafiqi et al., 2018). A transgenic non-antibiotic *E. coli* expressing *E. tenella* refractile protein SO7 (r*Et*SO7) was engineered, and its preventive capacity against homologous contagion was examined in three-day-old chickens (Yang et al., 2010). The chickens immunized with the r*Et*SO7 showed significantly immune protection in serum antibody, CD4(+) and CD8(+) T cells in peripheral blood of chickens characterized by an increase in body weight, decreased lesion scores and oocyst desquamation in the vaccinated chickens as against the controls (Yang et al., 2010). The report also indicated that the transgenic non-antibiotic *E. coli* that expresses the *Et*SO7 protein effectively stimulates host protective immunity characterized by the initiation of improvement of both humoral and cell-mediated immune outcomes against homologous challenge in chickens (Yang et al., 2010).

The immune response and preventive potential of *E. tenella* recombinant refractile protein (r*Et*SO7) in broiler chickens was evaluated following homologous oocyst challenge (Rafiqi et al., 2018). The outcome showed that r*Et*SO7 protein could effectively induce the cellular and humoral immune responses in immunized broiler chickens and could provide significant immune protection against caeca coccidiosis in chickens (Rafiqi et al., 2018). Broiler chickens immunized r*Et*SO7 showed a significant reduction in oocyst production and enhanced body weight gain, and an increase in IFN-γ, serum IgY levels and proliferation of lymphocytes were significant in broiler chickens vaccinated with r*Et*SO7 compared to the control (Rafiqi et al., 2018).

Rhoptry neck proteins (RON) are secreted early during parasite's intrusion and facilitates movement between parasite and host membranes (Oakes et al., 2013). Proteomic analysis of rhoptry organelles of *E. tenella* indicated *Et*RON2 was expressed in the sporozoitic and merozoitic stages while *Et*AMA1 was found only in the merozoite (Lal et al., 2009). However, a similar study showed that the orthologue of *Et*RON2 is explicit in both sporozoites and merozoites (Oakes et al., 2013).

Gametocyte (Gam) proteins are components of the oocysts cell wall presumed to be probable vaccine prospect against *Eimeria* in chicken. *Eimeria tenella* gametocyte (*Et*Gam22) protein generate a different and robust immune reaction affecting multiple cytokines and strong antibody titres, and it is a powerful immunogenic agent for use as a subunit immunizing agent against *Eimeria* coccidiosis in chickens (Rafiqi et al., 2019). Rafiqi *et al*., 2019 evaluated the immunoprophylactic potential of recombinant *Et*Gam22 antigen in broiler chickens against homologous oocysts dispute. Subcutaneous vaccination of broiler chickens with the transgenic *Et*Gam22 complexed with Montanide ISA 71 VG for two weeks, boosted by sporulated oocysts of *E. tenella* challenge for another one week, showed a significant increase in post-immunization (in the immunized broiler chickens), characterized with increase in anti-*Et*Gam22 IgY antibody, IL-2, TGF-β, and IL-4 serum and IFN-γ, levels (Rafiqi et al., 2019). The authors also reported that the peripheral blood lymphocyte increment activity of broiler chicken pre-challenge with *Et*Gam22 was significantly higher, while those immunized with the recombinant *Et*Gam22 (r*Et*Gam22) showed 45.23% reduction in oocyst output and higher weight gain per broiler (Rafiqi et al., 2019).

Liu *et al*., 2017 carried out the immunoproteomic investigation of the sporozoite proteins of *E. tenella*, *E. acervulina* and *E. maxima* to determine *Eimeria* sporozoites immunodominant antigen map, immunodominant antigens, and common immunodominant antigens for the improvement of multivalent anticoccidial immunizing agents (Liu et al., 2017). The analysis classified glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eukaryotic elongation factor 1 and 2 (EF 1 and EF 2), ubiquitin-conjugating enzyme domain-containing protein (UCE) and 14-3-3 proteins as immunodominant antigens from the eighteen ortholog proteins (common immunodominant antigens), out of the forty-four immunodominant proteins identified from *E. tenella*, *E. acervulina* and *E. maxima* respectively for developing multivalent anticoccidial vaccines (Liu et al., 2017).

Immunogenicity and immunoprotective properties of recombinant GAPDH proteins have been elucidated (Tian et al., 2017). The GAPDH protein elicits a fundamental humoral and cellular immune outcome and effectively protect chickens challenged infection by *E. tenella*, *E. acervulina*, *E. maxima*, and co-infection infection of the three *Eimeria* species (Tian et al., 2017).

Immunogenicity and immune protective potential of ubiquitin-conjugating enzyme domain-containing protein (UCE) against multiple *Eimeria* infections in chickens have also been examined by vaccination of chickens with recombinant UCE and challenge with *E. tenella*, *E. acervulina*, *E. maxima* and mixtures of the three *Eimeria* species, respectively (Liu et al., 2018). The recombinant UCE protein produces a significant humoral and cellular immune response and partially protect the chicken against *E. tenella*, *E. acervulina*, *E. maxima* and co-infection by the three *Eimeria* species and could be a potential antigen for the improvement of a multivalent immunizing agent against co-infections of multiple *Eimeria* species in domestic fowl industries (Liu et al., 2018).

Elongation constituents are concerned with bringing in aminoacyl-transfer RNA to the ribosome during protein synthesis and participate in the translocation of peptidyl-tRNA from one ribosomal site to another as the mRNA relocates across the ribosome (Hughes, 2013; Ling and Ermolenko, 2016).

The immunogenic potential of recombinant *E. tenella* elongation Factor-1α (r*Et*EF-1α) expressed in *E. coli* was examined in broiler chickens against *E. tenella* or *E. maxima* challenge. The broiler chickens immunized with r*Et*EF-1α demonstrated some level of immunity against *E. tenella* and *E. maxima* infections with enlarged body weight, reduced faecal oocyst production, and higher serum anti-EF-1α antibody levels against challenge infection with either *E. tenella* or *E. maxima* compared to unimmunized chickens (Lin et al., 2017). Immunization of broiler chickens with r*Et*EF-1α may be a new tool for producing cross-protective immunity against avian coccidiosis in the field (Lin et al., 2017). In another study, *Et*EF-1α co-administered with chicken cytokine IL-7 DNA vaccine (emulsified in Montanide Gel 01) enhanced the immune response to *E. acervulina* infection in broiler chickens (Panebra and Lillehoj, 2019).

Zhou *et al*., 2019 determined the role of *E. tenella* elongation factor 2 (*Et*EF 2) in diclazuril inhibition in second-generation merozoites of *E. tenella*. Diclazuril up-regulated mRNA and protein expression level of *Et*EF 2 and the protein localized primarily in the cytoplasm of second-generation merozoites. It may have an essential role in understanding the signalling mechanism underpinning the anticoccidial action of diclazuril and could be a potential tool for novel anticoccidial drug exploration (Zhou et al., 2019).

2.8 Antigenic diversity of *Et*AMA1 and its relevance to novel vaccine development

An antigen or antigenic agent is any substance that the immune system recognizes (as foreign), reacts with, and elicits an immune response against (Delves, 2020; Pichler et al., 2011). An antigen can be microorganisms (fungi, bacteria, viruses, alga, parasites, etc.), cancer cells, food molecules, pollens, self, toxins, or components of these agents. The ability of an antigenic agent to be recognized by the specific antibodies produced following the immune response to that agent is the antigenicity of that substance. In contrast, the ability of an antigenic agent to elicit both cellular and humoral immune reaction is the immunogenicity of an antigen (Ilinskaya and Dobrovolskaia, 2016). While both are often used interchangeably, it has been established that not all antigenic agents are immunogenic while all immunogenic agents are antigenic (Ilinskaya and Dobrovolskaia, 2016).

Antigenic diversity is known as a feature of various classes of infectious agent such as protozoans (Smith et al., 2002; Blake et al., 2015), viruses (Akashi and Inaba, 1997; Smith et al., 2002), and bacteria (Asanovich et al., 1997). There are dual mechanisms of antigenic diversity; the first mechanism is an antigenic modification where a clonal lineage of parasites expresses variant forms of an antigen without altering the genetic constitution (Ferreira et al., 2004). This means that an infection caused by a clonal lineage of *Eimeria* will not provide cross-protection in another clonal population challenged with another *Eimeria* species (Suprihati and Yunus, 2018). The intermediate process relates to the existence of genetically unfluctuating alternate forms of antigen-coding genes; this involves the classical genetic mechanism of nucleotide substitution and recombination that forms allelic polymorphism, thereby promoting persisters to thrive within the host in the proximity of immune system attack (Smith et al., 2002; Ferreira et al., 2004; Pastor-Fernández et al., 2018).

Antigenic variation helps pathogens elude the host immune reaction by continuously switching surface proteins (Sheykhsaran et al., 2019). Pathogens can avoid being detected by the host immune system by altering their antigenic targets; thus, the host immune system has become a significant target and determinant of pathogens' antigenic diversity (Georgieva et al., 2019).

Antigenic polymorphism is a standard mechanism used by many pathogens to circumvent the host immune system and presents significant challenges in vaccine development (Terheggen et al., 2014). Other research on antigenic diversity of key antigens reported that the consequences of antigenic polymorphism on potential vaccine evasion and how to sequence polymorphism related to antigenic diversity is minimal yet crucial for vaccine development (Terheggen et al., 2014). Furthermore, antigenic diversity may arise via hypermutation (Brunham et al., 1993) gene conversion (Pays et al., 1983) genetic recombination of sequence cassettes (Zhang et al., 1997) and site-specific DNA inversions (Lysnyansky et al., 2001) .This diversity makes some clonal populations of pathogens express heterologous phenotypes (Avery, 2006). Moreover, some of the proteins showing antigenic diversity or variation are linked to virulence (Van Der Woude and Bäumler, 2004).

The complex life cycle and inherent genetic polymorphism among *Eimeria* species make the production of recombinant anticoccidial vaccines challenging (Pastor-Fernández et al., 2018). *Eimeria* species produce between 6000 and 9000 antigens (Reid et al., 2014). Therefore, antigenic materials (surrogates) are incorporated into anticoccidial vaccine formulation to compensate for the naturally occurring genetic diversity of *Eimeria* (Venkatas and Adeleke, 2019). To accurately predict the potency and live span of subunit vaccines in field populations, the population constitution, probability of co-infection by genetically well-defined strains, pre-existing antigenic diversity, and the efficiency of cross-fertilization are essential; however, none of these four criteria is available for *E. tenella* (Blake et al., 2015).

Antigenic diverseness has been reported to underscore the efficacy of many experimental immunizing agents designed explicitly for apicomplexans (Arnott et al., 2014). To date, comprehensive sequence analysis to determine antigenic multifariousness has been carried out for only a few numbers of prospective vaccine antigens (Blake et al., 2017). *Et*AMA1 had been demonstrated to have high immune production and has been delineated as a prospective anticoccidial vaccine candidate under experimental conditions (Blake et al., 2011; Jiang et al., 2012).

Nevertheless, the pre-existing antigenic diversity and inharmonious population structure in other apicomplexans such as *Plasmodium* species have hampered vaccine improvement (Blake et al., 2015). The DNA sequence of monoclonal *Eimeria* populations in Northern and Southern India signalled advanced levels of genomic multifariousness within the AMA1 gene (Blake et al., 2015). However, genotypic profiling of *Et*AMA1 found high region-specific modification in haplotype variety for *Eimeria tenella* but low level of allelic polymorphism within *Et*AMA1 gene and the degraded level of *Et*AMA1 diversity enhance immunizing agent improvement (Blake et al., 2015). The low level of diversity within the *Et*AMA1 gene was also attributed to a lack of migration and interbreeding (Blake et al., 2015).

Furthermore, comparative investigation of the *Et*AMA1 coding sequence from field representatives of *E. tenella* strains of China, Egypt, Germany, India, Japan, Libya, Nigeria, UK, USA, and Venezuela, indicated an unassuming level of genetic polymorphism *Et*AMA1 across *E. tenella* strains from different geographical zones, but this is contrary to the genome-wide multifariousness studies for *E. tenella* (Blake et al., 2015). Sequence examination between states and single poultry farms showed no evidence of allopatric selection with multiple AMA1 sequences perceived on various farms. Only two out of the seven amino acid isoforms detected across the 56 AMA1 sequences were unique to specific geographical location (Blake et al., 2017). Analysis of the coding sequences polymorphisms (via Tajima's D and Fu and Li′s F* tests of neutrality) indicated no significant signatures of balancing or directional selection, supporting a neutral evolution (Blake et al., 2015).

Blake *et al*., 2015 also presumed that anticoccidial resistance *Eimeria* strains might spread rapidly through poultry farms in the event of emergence. They also alluded that *Eimeria* AMA1 retains its efficacy as anticoccidial immunizing agent candidates, based on the restricted genetic and antigenic multifariousness ascertained and a conservative signature of selection (Blake et al., 2015). The unelaborated nature of the immune protection provided by AMA1 vaccination may be advantageous to immunizing agent longevity by allowing local field strains to cycle at a level capable of assisting host immune protective cover and thinned genetic selection for immunizing agent resistance in a manner parallel to the attainment of ionophore based chemoprophylaxis (Chapman, 2014; Chapman and Jeffers, 2014). However, immune escape has been reported among *E. tenella* strains as an implication of apparent antigenic diversity (Awad et al., 2013) , though the escape level is deficient in outbred chicken lines (Blake et al., 2017). It is believed that mixtures of two or more antigenic agents are likely to help control each target *Eimeria* species (Blake et al., 2015).

Further, immune mapped protein-1 (IMP 1) has previously been characterized as a novel highly protective antigen and a potential anticoccidial vaccine candidate (Blake et al., 2011), and allelic polymorphism among the IMP 1 gene sequence in *E. tenella* strains from the USA, India, China, and the UK were compared with the UK reference (Houghton) strain (Kundu et al., 2017). The study found low nucleotide diversity across *Et*IMP1 gene sequences in *E. tenella* strains across the four countries. The observed diversity was restricted to the contraction and expansion of a CAG triplet repeats and five nucleotides substitutions, out of which three of which were non-synonymous (Kundu et al., 2017). The low antigenic diversity reported for *Et*AMA1 and *Et*IMP1 aligns with the ongoing development of these subunit types of proteins for inclusion in novel anticoccidial vaccines (Blake et al., 2017).

Despite these reports, there is no indication that immunizing agent resistance has developed in reaction to whole live parasite immunization (Shirley et al., 2005; Blake and Tomley, 2014). Strategies involving many immunoprotective antigens during replication in the hosts are likely to hinder the capability of any parasite to circumvent the host immune reaction as an aftermath of modifying selection (Blake et al., 2015; Clark et al., 2017). Therefore, the complexity of the antigenic repertory might suggest the reason why resistance to live parasite immunization is yet to emerged (Blake and Tomley, 2014; Blake et al., 2015).

2.9 Novel vaccine design through immunoinformatics

Vaccine improvement and manufacture are expensive, and it takes years for vaccine production to be achieved (María et al., 2017). Several alternative plans have been developed to cut back the time and costs of immunizing agent improvement by concentrating on selecting befitting antigens or antigenic constitutes, carriers, and adjuvants (María et al., 2017). One of these conceptualizations is integrating bioinformatics approaches and analyses into immunizing agent design and modification (María et al., 2017). There are many schemes that are currently used to plan and evolve impelling and unhazardous new-generation immunizing agent, founded on bioinformatics conceptualization via immunoinformatics, reverse vaccinology, and composition vaccinology (Seib et al., 2012; María et al., 2017). Immunoinformatics strategy is based on the use of different software packages. This software is based on machine learning such as position-specific scoring matrices (PSSMs), support vector machines (SVMs), hidden Markov models (HMMs), or artificial neural networks (ANNs)} with epitopes and non-epitopes previously characterized to predict whether or not it is an epitope (María et al., 2017).

Immunoinformatics (a combination of bioinformatics and immunomics) was created to analyze all of the information of microorganism's immunomics and make predictions of immune responses against specific and/or target molecules/proteins (Flower, 2009; María et al., 2017). Different websites and data repositories such as AntiJen (Toseland et al., 2005) IEDB (Fleri et al., 2017), SIFPEITHI (Rammensee et al., 1999), IMGT (Lefranc et al., 2015) MHCBN (Lata et al., 2009), Dana-Farber Repository (Zhang et al., 2011), and AgAbDb (Kulkarni-Kale et al., 2014) that present databases of antigens with their epitopes identified in various organisms, and other immunological subject matter have also been developed.

Once an antigen/protein with the awaited immune response has been recognized and known, immunoinformatics can be used to foretell whether a region of that antigen/protein can elicit sufficient self-stimulus (María et al., 2017). Suppose such antigen/protein has one epitope. In that case, it can be used in a subunit immunizing agent development or can be compounded with other epitopes from different sources to develop a polyvalent immunizing agent, thereby reducing the price of the production (María et al., 2017).

Immunoinformatics strategies have been used to identify conserved sequences in assorted viral proteins such as Ebola virus nucleoprotein (Ali and Islam, 2015) and glycoprotein (Dash et al., 2017), Zika virus glycoprotein (Dikhit et al., 2016), Chikungunya virus proteins, Nipah virus fusion and glycoprotein (Ali et al., 2015), nucleocapsid and glycoprotein of Rift Valley fever virus (Adhikari and Rahman, 2017). The multiepitope vaccine against *Mycobacterium ulcerans* has also been designed using integrated advanced vaccinomic methods (Nain et al., 2019).

Immunoinformatics approach has been used to identify several MERS-COVS protein epitopes that are conserved amid respective isolates of middle east respiratory syndrome coronavirus from assorted nations using peptide-based vaccine design and target site characterization (Tahir Ul Qamar et al., 2019). Other studies have also designed a multiepitope vaccine (which could stimulate the humoral and cellular immune response in mice) against the 2019 novel coronavirus (SARS-COV_2) using the immunoinformatic strategy (Naz et al., 2020; Sanami et al., 2020).

Furthermore, the immunoinformatics initiative has also been used to design a peptide-based subunit vaccine of T- and B-Cells multi-epitopes against the Zika Virus (Prasasty et al., 2019). This technique has also been used to design and select *Plasmodium falciparum* epitope ensemble vaccines, and the selected epitopes were predicted to give 97.9% universal coverage

(Damfo et al., 2017; Pritam et al., 2019). However, little or no study is available on the application of this novel technique in the control of *Eimeria* infection in chicken.

2.10 Conclusion

Anticoccidial vaccines prepared using live *Eimeria* species are an efficient alternative to chemoprophylaxis as the consumption and recycling of controlled dosage of immunizing agent oocysts elicits robust immune protection. Live anticoccidial vaccines have been used in small facets of the commercial poultry enterprise for more than 50 years with no information of parasite development toward resistance/immune escape. Naturally present genetic multifariousness has been dependably surmounted by including more than one strain of a single species in some immunizing agent formulations. However, these live vaccines have to be grown in chickens.

Furthermore, the low reproductive index of decreased immunizing agent parasites makes the costs of production higher and also constricts capacity. Production of live wild-type and attenuated anticoccidial immunizing agents is also time-consuming and is restricted by the need for in vivo infection of the chickens to produce infectious oocysts, which cannot be obtained in vitro. Additionally, cost-effective subunit vaccines, consisting of a small number of *Eimeria* antigens, are required for the mass broiler marketplace, but there are still many requirements to be met to ensure their complete efficacy against *Eimeria* infection. Overall, existent vaccines are comparatively costly due to high manufacturing costs and are challenging to scale up, particularly attenuated parasite lines.

Eimeria antigenic agents are potential vaccine candidates supported by their host-parasite intrusion, interaction, and multiplication. Immunoinformatics strategies have been used to identify conserved sequences in different viral and bacterial proteins. It can also be used to design a robust polyvalent coccidiosis vaccine that can be used in all poultry farms. It is believed that the use of immunoinformatics strategies could be a promising alternative method of designing an effective multiepitope vaccine against coccidiosis.

2.11 References

Abdelli, N., Pérez, J.F., Vilarrasa, E., Luna, I.C., Melo-Duran, D., D'angelo, M., Solà-Oriol, D., 2020. Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge. Animals 10. https://doi.org/10.3390/ani10020259

Adhikari, U.K., Rahman, M.M., 2017. Overlapping CD8 + and CD4 + T-cell epitopes identification for the progression of epitope-based peptide vaccine from nucleocapsid and glycoprotein of emerging Rift Valley fever virus using immunoinformatics approach. Infect. Genet. Evol. 56. https://doi.org/10.1016/j.meegid.2017.10.022

Ahmad, T.A., El-Sayed, B.A., El-Sayed, L.H., 2016. Development of immunization trials against Eimeria spp. Trials Vaccinol. https://doi.org/10.1016/j.trivac.2016.02.001

Akashi, H., Inaba, Y., 1997. Antigenic diversity of Akabane virus detected by monoclonal antibodies. Virus Res. 47. https://doi.org/10.1016/S0168-1702(96)01415-3

Al-Mamun, M., Islam, K., Rahman, M., 2019. Occurrence of poultry diseases at Kishoregonj district of Bangladesh. MOJ Proteomics Bioinforma. 8.

Ali, M.T., Islam, M.O., 2015. A highly conserved GEQYQQLR epitope has been identified in the nucleoprotein of Ebola virus by using an in silico approach. Adv. Bioinformatics 2015. https://doi.org/10.1155/2015/278197

Ali, M.T., Morshed, M.M., Hassan, F., 2015. A Computational Approach for Designing a Universal Epitope-Based Peptide Vaccine Against Nipah Virus. Interdiscip. Sci. Comput. Life Sci. 7. https://doi.org/10.1007/s12539-015-0023-0

Allen, P.C., Fetterer, R.H., 2002. Recent advances in biology and immunobiology of Eimeria species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.15.1.58-65.2002

Arafat, N., Abbas, I., 2018. Coccidia of Japanese Quail: From Identification, Prevalence, Infection, and Immunization. J. Parasitol. 104. https://doi.org/10.1645/17-109

Arnott, A., Wapling, J., Mueller, I., Ramsland, P.A., Siba, P.M., Reeder, J.C., Barry, A.E., 2014. Distinct patterns of diversity, population structure and evolution in the AMA1 genes of sympatric Plasmodium falciparum and Plasmodium vivax populations of Papua New Guinea from an area of similarly high transmission. Malar. J. 13. https://doi.org/10.1186/1475-2875-13-233

Asanovich, K.M., Bakken, J.S., Madigan, J.E., Aguero-Rosenfeld, M., Wormser, G.P., Dumler, J.S., 1997. Antigenic diversity of granulocytic Ehrlichia isolates from humans in Wisconsin and New York and a horse in California. J. Infect. Dis. 176. https://doi.org/10.1086/516529

Avery, S. V., 2006. Microbial cell individuality and the underlying sources of heterogeneity. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro1460

Awad, A.M., El-Nahas, A.F., Abu-Akkada, S.S., 2013. Evaluation of the protective efficacy of the anticoccidial vaccine Coccivac-B in broilers, when challenged with Egyptian field isolates of E. tenella. Parasitol. Res. 112, 113–121. https://doi.org/10.1007/s00436-012-3112-6

Bafundo, K.W., Cervantes, H.M., Mathis, G.F., 2008. Sensitivity of Eimeria field isolates in

the United Sstates: Responses of nicarbazin-containing anticoccidials. Poult. Sci. 87. https://doi.org/10.3382/ps.2008-00129

Barkway, C.P., Pocock, R.L., Vrba, V., Blake, D.P., 2015. Loop-mediated isothermal amplification (LAMP) assays for the species-specific detection of Eimeria that infect chickens. J. Vis. Exp. https://doi.org/10.3791/52552

Belete, A., Addis, M., Ayele, M., 2016. Review on major gastrointestinal parasites that affect chickens. J. Biol. Agric. Healthc. 6.

Blake, D.P., Billington, K.J., Copestake, S.L., Oakes, R.D., Quail, M.A., Wan, K.L., Shirley, M.W., Smith, A.L., 2011. Genetic mapping identifies novel highly protective antigens for an apicomplexan parasite. PLoS Pathog. 7. https://doi.org/10.1371/journal.ppat.1001279

Blake, D.P., Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Jatau, I.D., Ayoade, S., Kawahara, F., Moftah, A., Reid, A.J., Adebambo, A.O., Zapata, R.Á., Rao, A.S.R.S., Thangaraj, K., Banerjee, P.S., Dhinakar-Raj, G., Raman, M., Tomley, F.M., 2015. Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. Proc. Natl. Acad. Sci. U. S. A. 112. https://doi.org/10.1073/pnas.1506468112

Blake, D.P., Hesketh, P., Archer, A., Carroll, F., Smith, A.L., Shirley, M.W., 2004. Parasite genetics and the immune host: Recombination between antigenic types of Eimeria maxima as an entrée to the identification of protective antigens. Mol. Biochem. Parasitol. 138. https://doi.org/10.1016/j.molbiopara.2004.08.006

Blake, D.P., Pastor-Fernández, I., Nolan, M.J., Tomley, F.M., 2017. Recombinant anticoccidial vaccines - a cup half full? Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2017.10.009

Blake, D.P., Tomley, F.M., 2014. Securing poultry production from the ever-present Eimeria challenge. Trends Parasitol. https://doi.org/10.1016/j.pt.2013.10.003

Blumenschein, T.M.A., Friedrich, N., Childs, R.A., Saouros, S., Carpenter, E.P., Campanero-Rhodes, M.A., Simpson, P., Chai, W., Koutroukides, T., Blackman, M.J., Feizi, T., Soldati-Favre, D., Matthews, S., 2007. Atomic resolution insight into host cell recognition by Toxoplasma gondii. EMBO J. 26. https://doi.org/10.1038/sj.emboj.7601704

Borgonovo, F., Ferrante, V., Grilli, G., Pascuzzo, R., Vantini, S., Guarino, M., 2020. A data-driven prediction method for an early warning of coccidiosis in intensive livestock systems: A preliminary study. Animals 10. https://doi.org/10.3390/ani10040747

Boulton, K., Nolan, M.J., Wu, Z., Psifidi, A., Riggio, V., Harman, K., Bishop, S.C., Kaiser, P., Abrahamsen, M.S., Hawken, R., Watson, K.A., Tomley, F.M., Blake, D.P., Hume, D.A., 2018. Phenotypic and genetic variation in the response of chickens to Eimeria tenella induced coccidiosis. Genet. Sel. Evol. 50. https://doi.org/10.1186/s12711-018-0433-7

Brown, P.J., Mulvey, D., Potts, J.R., Tomley, F.M., Campbell, I.D., 2003. Solution structure of a PAN module from the apicomplexan parasite Eimeria tenella. J. Struct. Funct. Genomics 4. https://doi.org/10.1023/B:JSFG.0000016118.87333.03

Brunham, R.C., Plummer, F.A., Stephens, R.S., 1993. Bacterial antigenic variation, host

immune response, and pathogen-host coevolution. Infect. Immun. https://doi.org/10.1128/iai.61.6.2273-2276.1993

Bumstead, J., Tomley, F., 2000. Induction of secretion and surface capping of microneme proteins in Eimeria tenella, Molecular and Biochemical Parasitology.

Chapman, H.D., 2014. Milestones in avian coccidiosis research: A review. Poult. Sci. https://doi.org/10.3382/ps.2013-03634

Chapman, H.D., 1999. Anticoccidial drugs and their effects upon the development of immunity to Eimeria infections in poultry. Avian Pathol. https://doi.org/10.1080/03079459994317

Chapman, H.D., 1997. Biochemical, genetic and applied aspects of drug resistance in Eimeria parasites of the fowl. Avian Pathol. https://doi.org/10.1080/03079459708419208

Chapman, H.D., Jeffers, T.K., 2014. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. Int. J. Parasitol. Drugs Drug Resist. 4. https://doi.org/10.1016/j.ijpddr.2014.10.002

Chen, H.L., Zhao, X.Y., Zhao, G.X., Huang, H. Bin, Li, H.R., Shi, C.W., Yang, W.T., Jiang, Y.L., Wang, J.Z., Ye, L.P., Zhao, Q., Wang, C.F., Yang, G.L., 2020. Dissection of the cecal microbial community in chickens after Eimeria tenella infection. Parasites and Vectors 13. https://doi.org/10.1186/s13071-020-3897-6

Chengat Prakashbabu, B., Thenmozhi, V., Limon, G., Kundu, K., Kumar, S., Garg, R., Clark, E.L., Srinivasa Rao, A.S.R., Raj, D.G., Raman, M., Banerjee, P.S., Tomley, F.M., Guitian, J., Blake, D.P., 2017. Eimeria species occurrence varies between geographic regions and poultry production systems and may influence parasite genetic diversity. Vet. Parasitol. 233. https://doi.org/10.1016/j.vetpar.2016.12.003

Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Kumar, S., Ayoade, S., Fornace, K.M., Jatau, I.D., Moftah, A., Nolan, M.J., Sudhakar, N.R., Adebambo, A.O., Lawal, I.A., Álvarez Zapata, R., Awuni, J.A., Chapman, H.D., Karimuribo, E., Mugasa, C.M., Namangala, B., Rushton, J., Suo, X., Thangaraj, K., Srinivasa Rao, A.S.R., Tewari, A.K., Banerjee, P.S., Dhinakar Raj, G., Raman, M., Tomley, F.M., Blake, D.P., 2016. Cryptic Eimeria genotypes are common across the southern but not northern hemisphere. Int. J. Parasitol. 46. https://doi.org/10.1016/j.ijpara.2016.05.006

Clark, E.L., Tomley, F.M., Blake, D.P., 2017. Are Eimeria Genetically Diverse, and Does It Matter? Trends Parasitol. https://doi.org/10.1016/j.pt.2016.08.007

Dalloul, R.A., Lillehoj, H.S., 2006. Poultry coccidiosis: Recent advancements in control measures and vaccine development. Expert Rev. Vaccines. https://doi.org/10.1586/14760584.5.1.143

Damfo, S.A., Reche, P., Gatherer, D., Flower, D.R., 2017. In silico design of knowledge-based Plasmodium falciparum epitope ensemble vaccines. J. Mol. Graph. Model. 78. https://doi.org/10.1016/j.jmgm.2017.10.004

Dash, R., Das, R., Junaid, M., Akash, M.F.C., Islam, A., Hosen, S.M.Z., 2017. In silico-based vaccine design against Ebola virus glycoprotein. Adv. Appl. Bioinforma. Chem. 10. https://doi.org/10.2147/AABC.S115859

Dikhit, M.R., Ansari, M.Y., Vijaymahantesh, Kalyani, Mansuri, R., Sahoo, B.R., Dehury, B., Amit, A., Topno, R.K., Sahoo, G.C., Ali, V., Bimal, S., Das, P., 2016. Computational

prediction and analysis of potential antigenic CTL epitopes in Zika virus: A first step towards vaccine development. Infect. Genet. Evol. 45. https://doi.org/10.1016/j.meegid.2016.08.037

Eckford, S., Grace, K., Harris, C., Reeves, H., 2013. UK Veterinary Antibiotic Resistance and Sales Surveillance: UK-VARSS 2013. UK Vet. Antibiot. Resist. Sales Surveill. UK VARSS 132.

FAOSTAT, 2016. (Food and Agriculture Organization of United Nations). 2016. http://www.fao.org/faostat/en/#data. [WWW Document].

Fatoba, A.J., Adeleke, M.A., 2018. Diagnosis and control of chicken coccidiosis: a recent update. J. Parasit. Dis. https://doi.org/10.1007/s12639-018-1048-1

Fernandez, S., Katsuyama, Â.M., Kashiwabara, A.Y., Madeira, A.M.B.N., Durham, A.M., Gruber, A., 2004. Characterization of SCAR markers of Eimeria spp. of domestic fowl and construction of a public relational database (The Eimeria SCARdb). FEMS Microbiol. Lett. 238. https://doi.org/10.1016/j.femsle.2004.07.034

Ferreira, M.U., Da Silva Nunes, M., Wunderlich, G., 2004. Antigenic diversity and immune evasion by malaria parasites. Clin. Diagn. Lab. Immunol. https://doi.org/10.1128/CDLI.11.6.987-995.2004

Fleri, W., Paul, S., Dhanda, S.K., Mahajan, S., Xu, X., Peters, B., Sette, A., 2017. The immune epitope database and analysis resource in epitope discovery and synthetic vaccine design. Front. Immunol. https://doi.org/10.3389/fimmu.2017.00278

Flower, D.R., 2009. Bioinformatics for Vaccinology, Bioinformatics for Vaccinology. https://doi.org/10.1002/9780470699836

Georgieva, M., Buckee, C.O., Lipsitch, M., 2019. Models of immune selection for multi-locus antigenic diversity of pathogens. Nat. Rev. Immunol. https://doi.org/10.1038/s41577-018-0092-5

Hafeez, M.A., Shivaramaiah, S., Dorsey, K.M., Ogedengbe, M.E., El-Sherry, S., Whale, J., Cobean, J., Barta, J.R., 2015. Simultaneous identification and DNA barcoding of six Eimeria species infecting turkeys using PCR primers targeting the mitochondrial cytochrome c oxidase subunit I (mtCOI) locus. Parasitol. Res. 114. https://doi.org/10.1007/s00436-015-4361-y

Haug, A., Gjevre, A.G., Thebo, P., Mattsson, J.G., Kaldhusdal, M., 2008. Coccidial infections in commercial broilers: Epidemiological aspects and comparison of Eimeria species identification by morphometric and polymerase chain reaction techniques. Avian Pathol. 37. https://doi.org/10.1080/03079450801915130

Hinsu, A.T., Thakkar, J.R., Koringa, P.G., Vrba, V., Jakhesara, S.J., Psifidi, A., Guitian, J., Tomley, F.M., Rank, D.N., Raman, M., Joshi, C.G., Blake, D.P., 2018. Illumina Next Generation Sequencing for the Analysis of Eimeria Populations in Commercial Broilers and Indigenous Chickens. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00176

Huang, X., Liu, J., Tian, D., Li, W., Zhou, Z., Huang, J., Song, X., Xu, L., Yan, R., Li, X., 2018. The molecular characterization and protective efficacy of microneme 3 of Eimeria mitis in chickens. Vet. Parasitol. 258. https://doi.org/10.1016/j.vetpar.2018.06.020

Hughes, D., 2013. Elongation Factors: Translation, in: Brenner's Encyclopedia of Genetics: Second Edition. Elsevier Inc., pp. 466–468. https://doi.org/10.1016/B978-0-12-374984-

0.00468-X

Ilinskaya, A.N., Dobrovolskaia, M.A., 2016. Understanding the immunogenicity and antigenicity of nanomaterials: Past, present and future. Toxicol. Appl. Pharmacol. 299, 70–77. https://doi.org/10.1016/j.taap.2016.01.005

Jenkins, M.C., O'Brien, C.N., Parker, C., 2019. Excystation of Eimeria acervulina, E. maxima, and E. tenella differs in response to trypsin and chymotrypsin and the presence of reducing agents DTT and TCEP. Mol. Biochem. Parasitol. 233. https://doi.org/10.1016/j.molbiopara.2019.111219

Jiang, L., Lin, J., Han, H., Dong, H., Zhao, Q., Zhu, S., Huang, B., 2012. Identification and characterization of eimeria tenella apical membrane antigen-1 (ama1). PLoS One 7. https://doi.org/10.1371/journal.pone.0041115

Johnson, J., Reid, W.M., 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol. 28. https://doi.org/10.1016/0014-4894(70)90063-9

Kulkarni-Kale, U., Raskar-Renuse, S., Natekar-Kalantre, G., Saxena, S.A., 2014. Antigen-Antibody Interaction Database (AgAbDb): A compendium of antigen-antibody interactions. Methods Mol. Biol. 1184. https://doi.org/10.1007/978-1-4939-1115-8_8

Kumar, S., Garg, R., Moftah, A., Clark, E.L., Macdonald, S.E., Chaudhry, A.S., Sparagano, O., Banerjee, P.S., Kundu, K., Tomley, F.M., Blake, D.P., 2014. An optimised protocol for molecular identification of Eimeria from chickens. Vet. Parasitol. 199. https://doi.org/10.1016/j.vetpar.2013.09.026

Kundu, K., Garg, R., Kumar, S., Mandal, M., Tomley, F.M., Blake, D.P., Banerjee, P.S., 2017. Humoral and cytokine response elicited during immunisation with recombinant Immune Mapped protein-1 (EtIMP-1) and oocysts of Eimeria tenella. Vet. Parasitol. 244. https://doi.org/10.1016/j.vetpar.2017.07.025

Kundu, K., Kumar, S., Banerjee, P.S., Garg, R., 2020. Quantification of Eimeria necatrix, E. acervulina and E. maxima genomes in commercial chicken farms by quantitative real time PCR. J. Parasit. Dis. 44. https://doi.org/10.1007/s12639-019-01188-2

Kvičerová, J., Hypša, V., 2013. Host-Parasite Incongruences in Rodent Eimeria Suggest Significant Role of Adaptation Rather than Cophylogeny in Maintenance of Host Specificity. PLoS One 8. https://doi.org/10.1371/journal.pone.0063601

Labbé, M., Péroval, M., Bourdieu, C., Girard-Misguich, F., Péry, P., 2006. Eimeria tenella enolase and pyruvate kinase: A likely role in glycolysis and in others functions. Int. J. Parasitol. 36. https://doi.org/10.1016/j.ijpara.2006.08.011

Lai, L., Bumstead, J., Liu, Y., Garnett, J., Campanero-Rhodes, M.A., Blake, D.P., Palma, A.S., Chai, W., Ferguson, D.J.P., Simpson, P., Feizi, T., Tomley, F.M., Matthews, S., 2011. The role of Sialyl glycan recognition in host tissue tropism of the avian parasite Eimeria Tenella. PLoS Pathog. 7. https://doi.org/10.1371/journal.ppat.1002296

Lai, L., Simpson, P., Bumstead, J., Tomley, F., Matthews, S., 2009. Complete NMR assignments for the second microneme adhesive repeat (MAR) domain from Eimeria tenella microneme protein EtMIC3. Biomol. NMR Assign. 3. https://doi.org/10.1007/s12104-009-9168-2

Lal, K., Bromley, E., Oakes, R., Prieto, J.H., Sanderson, S.J., Kurian, D., Hunt, L., Yates,

J.R., Wastling, J.M., Sinden, R.E., Tomley, F.M., 2009. Proteomic comparison of four Eimeria tenella life-cycle stages: Unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. Proteomics 9. https://doi.org/10.1002/pmic.200900305

Lata, S., Bhasin, M., Raghava, G.P., 2009. MHCBN 4.0: A database of MHC/TAP binding peptides and T-cell epitopes. BMC Res. Notes 2. https://doi.org/10.1186/1756-0500-2-61

Lefranc, M.P., Giudicelli, V., Duroux, P., Jabado-Michaloud, J., Folch, G., Aouinti, S., Carillon, E., Duvergey, H., Houles, A., Paysan-Lafosse, T., Hadi-Saljoqi, S., Sasorith, S., Lefranc, G., Kossida, S., 2015. IMGT R, the international ImMunoGeneTics information system R 25 years on. Nucleic Acids Res. 43. https://doi.org/10.1093/nar/gku1056

Li, C., Zhao, Q., Zhu, S., Wang, Q., Wang, H., Yu, S., Yu, Y., Liang, S., 2020. Eimeria tenella Eimeria - specific protein that interacts with apical membrane antigen 1 (Et AMA1) is involved in host cell invasion 1–13. https://doi.org/10.1186/s13071-020-04229-5

Li, G., Ma, C., Wang, D., Chen, W., Ma, D., 2020. Recombinant Lactococcus lactis co-expressing dendritic cell target peptide and E. tenella 3-1E protein: immune response and efficacy against homologous challenge. Food Agric. Immunol. 31. https://doi.org/10.1080/09540105.2020.1733495

Li, J., Wang, F., Ma, C., Huang, Y., Wang, D., Ma, D., 2018. Recombinant lactococcus lactis expressing Eimeria tenella AMA1 protein and its immunological effects against homologous challenge. Exp. Parasitol. 191. https://doi.org/10.1016/j.exppara.2018.05.003

Lillehoj, H.S., Jang, S.I., Panebra, A., Lillehoj, E.P., Dupuis, L., Arous, J. Ben, Lee, S.K., Oh, S.T., 2017. In ovo vaccination using Eimeria profilin and Clostridium perfringens NetB proteins in Montanide IMS adjuvant increases protective immunity against experimentally-induced necrotic enteritis. Asian-Australasian J. Anim. Sci. 30. https://doi.org/10.5713/ajas.17.0053

Lin, R.Q., Lillehoj, H.S., Lee, S.K., Oh, S., Panebra, A., Lillehoj, E.P., 2017. Vaccination with Eimeria tenella elongation factor-1α recombinant protein induces protective immunity against E. tenella and E. maxima infections. Vet. Parasitol. 243. https://doi.org/10.1016/j.vetpar.2017.06.003

Ling, C., Ermolenko, D.N., 2016. Structural insights into ribosome translocation. Wiley Interdiscip. Rev. RNA 7. https://doi.org/10.1002/wrna.1354

Liu, J., Liu, L., Li, L., Tian, D., Li, W., Xu, L., Yan, R., Li, X., Song, X., 2018. Protective immunity induced by Eimeria common antigen 14-3-3 against Eimeria tenella, Eimeria acervulina and Eimeria maxima 11 Medical and Health Sciences 1107 Immunology. BMC Vet. Res. 14. https://doi.org/10.1186/s12917-018-1665-z

Liu, L., Huang, X., Liu, J., Li, W., Ji, Y., Tian, D., Tian, L., Yang, X., Xu, L., Yan, R., Li, X., Song, X., 2017. Identification of common immunodominant antigens of Eimeria tenella, Eimeria acervulina and Eimeria maxima by immunoproteomic analysis. Oncotarget 8. https://doi.org/10.18632/oncotarget.16824

Liu, Q., Chen, Z., Shi, W., Sun, H., Zhang, J., Li, H., Xiao, Y., Wang, F., Zhao, X., 2014. Preparation and initial application of monoclonal antibodies that recognize Eimeria

tenella microneme proteins 1 and 2. Parasitol. Res. 113. https://doi.org/10.1007/s00436-014-4087-2

Long, P.L., Millard, B.J., Batty, A.F., Davison, C., 1982. Immunisation against coccidiosis in chickens: Tests under simulated field conditions. Avian Pathol. 11. https://doi.org/10.1080/03079458208436088

Lysnyansky, I., Ron, Y., Yogev, D., 2001. Juxtaposition of an active promoter to vsp genes via site-specific DNA inversions generates antigenic variation in Mycoplasma bovis. J. Bacteriol. 183. https://doi.org/10.1128/JB.183.19.5698-5708.2001

Macdonald, S.E., Nolan, M.J., Harman, K., Boulton, K., Hume, D.A., Tomley, F.M., Stabler, R.A., Blake, D.P., 2017. Effects of Eimeria tenella infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. PLoS One 12. https://doi.org/10.1371/journal.pone.0184890

María, R.R., Arturo, C.J., Alicia, J.A., Paulina, M.G., Gerardo, A.O., 2017. The Impact of Bioinformatics on Vaccine Design and Development, in: Vaccines. InTech. https://doi.org/10.5772/intechopen.69273

Marugan-Hernandez, V., Cockle, C., Macdonald, S., Pegg, E., Crouch, C., Blake, D.P., Tomley, F.M., 2016. Viral proteins expressed in the protozoan parasite Eimeria tenella are detected by the chicken immune system. Parasites and Vectors 9. https://doi.org/10.1186/s13071-016-1756-2

McDonald, V., Ballingall, S., 1983. Further Investigation of the Pathogenicity, Immunogenicity and Stability of Precocious Eimeria Acervulina. Parasitology 86. https://doi.org/10.1017/S0031182000050551

McDougald, L.R., Cervantes, H.M., Jenkins, M.C., Hess, M., Beckstead, R., 2019. Protozoal infections, in: Diseases of Poultry. https://doi.org/10.1002/9781119371199.ch28

Miska, K.B., Schwarz, R.S., Jenkins, M.C., Rathinam, T., Chapman, H.D., 2010. Molecular characterization and phylogenetic analysis of Eimeria from Turkeys and Gamebirds: Implications for evolutionary relationships in Galliform birds. J. Parasitol. 96. https://doi.org/10.1645/GE-2344.1

Mohammad, M.H., Ahad, O., Mohammad, N., Fariborz, A., Omid, N., 2011. Prevalence of Eimeria species in scavenging native chickens of Shiraz, Iran. African J. Microbiol. Res. 5. https://doi.org/10.5897/ajmr11.477

Moore, R.J., 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathol. https://doi.org/10.1080/03079457.2016.1150587

Morris, G.M., Gasser, R.B., 2006. Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in Eimeria. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2006.06.001

Nain, Z., Karim, M.M., Sen, M.K., Adhikari, U.K., 2019. Structural basis and designing of peptide vaccine using PE-PGRS family protein of mycobacterium ulcerans - An integrated vaccinomics approach. bioRxiv. https://doi.org/10.1101/795146

Narasinakuppe K., D., Dhama, K., Kumar Mariappan, A., Munuswamy, P., Iqbal Yatoo, M., Tiwari, R., Karthik, K., Bhatt, P., Reddy, M.R., 2020. Etiology, epidemiology, pathology, and advances in diagnosis, vaccine development, and treatment of Gallibacterium anatis infection in poultry: a review. Vet. Q.

https://doi.org/10.1080/01652176.2020.1712495

Naz, A., Shahid, F., Butt, T.T., Awan, F.M., Ali, A., Malik, A., 2020. Designing Multi-Epitope Vaccines to Combat Emerging Coronavirus Disease 2019 (COVID-19) by Employing Immuno-Informatics Approach. Front. Immunol. 11. https://doi.org/10.3389/fimmu.2020.01663

Oakes, R.D., Kurian, D., Bromley, E., Ward, C., Lal, K., Blake, D.P., Reid, A.J., Pain, A., Sinden, R.E., Wastling, J.M., Tomley, F.M., 2013. The rhoptry proteome of Eimeria tenella sporozoites. Int. J. Parasitol. 43. https://doi.org/10.1016/j.ijpara.2012.10.024

Panebra, A., Lillehoj, H.S., 2019. Eimeria tenella Elongation Factor-1α (EF-1α) Coadministered with Chicken IL-7 (chIL-7) DNA Vaccine Emulsified in Montanide Gel 01 Adjuvant Enhanced the Immune Response to E. acervulina Infection in Broiler Chickens. Avian Dis. 63. https://doi.org/10.1637/11976-092418-Reg.1

Parker, M.L., Penarete-Vargas, D.M., Hamilton, P.T., Guérin, A., Dubey, J.P., Perlman, S.J., Spano, F., Lebrun, M., Boulanger, M.J., 2016. Dissecting the interface between apicomplexan parasite and host cell: Insights from a divergent AMA-RON2 pair. Proc. Natl. Acad. Sci. U. S. A. 113. https://doi.org/10.1073/pnas.1515898113

Pastor-Fernández, I., Kim, S., Billington, K., Bumstead, J., Marugán-Hernández, V., Küster, T., Ferguson, D.J.P., Vervelde, L., Blake, D.P., Tomley, F.M., 2018. Development of cross-protective Eimeria-vectored vaccines based on apical membrane antigens. Int. J. Parasitol. 48. https://doi.org/10.1016/j.ijpara.2018.01.003

Pays, E., Van Assel, S., Laurent, M., Darville, M., Vervoort, T., Van Meirvenne, N., Steinert, M., 1983. Gene conversion as a mechanism for antigenic variation in Trypanosomes. Cell 34. https://doi.org/10.1016/0092-8674(83)90371-9

Peek, H.W., Landman, W.J.M., 2011. Coccidiosis in poultry: Anticoccidial products, vaccines and other prevention strategies. Vet. Q. 31. https://doi.org/10.1080/01652176.2011.605247

Periz, J., Gill, A.C., Hunt, L., Brown, P., Tomley, F.M., 2007. The microneme proteins EtMIC4 and EtMIC5 of Eimeria tenella form a novel, ultra-high molecular mass protein complex that binds target host cells. J. Biol. Chem. 282. https://doi.org/10.1074/jbc.M702407200

Periz, J., Gill, A.C., Knott, V., Handford, P.A., Tomley, F.M., 2005. Calcium binding activity of the epidermal growth factor-like domains of the apicomplexan microneme protein EtMIC4. Mol. Biochem. Parasitol. 143. https://doi.org/10.1016/j.molbiopara.2005.06.003

Periz, J., Ryan, R., Blake, D.P., Tomley, F.M., 2009. Eimeria tenella microneme protein EtMIC4: Capture of the full-length transcribed sequence and comparison with other microneme proteins. Parasitol. Res. 104. https://doi.org/10.1007/s00436-008-1301-0

Pichler, W.J., Naisbitt, D.J., Park, B.K., 2011. Immune pathomechanism of drug hypersensitivity reactions, in: Journal of Allergy and Clinical Immunology. https://doi.org/10.1016/j.jaci.2010.11.048

Prasasty, V.D., Grazzolie, K., Rosmalena, R., Yazid, F., Ivan, F.X., Sinaga, E., 2019. Peptide-based subunit vaccine design of T-and b-cells multi-epitopes against zika virus using immunoinformatics approaches. Microorganisms 7.

https://doi.org/10.3390/microorganisms7080226

Price, K.R., Guerin, M.T., Barta, J.R., 2014. Success and failure: The role of relative humidity levels and environmental management in live Eimeria vaccination of cage-reared replacement layer pullets. J. Appl. Poult. Res. 23. https://doi.org/10.3382/japr.2014-00989

Price, K.R., Hafeez, M.A., Bulfon, J., Barta, J.R., 2016. Live Eimeria vaccination success in the face of artificial non-uniform vaccine administration in conventionally reared pullets. Avian Pathol. 45. https://doi.org/10.1080/03079457.2015.1125442

Pritam, M., Singh, G., Swaroop, S., Singh, A.K., Singh, S.P., 2019. Exploitation of reverse vaccinology and immunoinformatics as promising platform for genome-wide screening of new effective vaccine candidates against Plasmodium falciparum. BMC Bioinformatics 19. https://doi.org/10.1186/s12859-018-2482-x

Rabenau, K.E., Sohrabi, A., Tripathy, A., Reitter, C., Ajioka, J.W., Tomley, F.M., Carruthers, V.B., 2001. TgM2AP participates in toxoplasma gondii invasion of host cells and is tightly associated with the adhesive protein TgMIC2. Mol. Microbiol. 41. https://doi.org/10.1046/j.1365-2958.2001.02513.x

Rafiqi, S.I., Garg, R., Ram, H., Reena, K.K., Asari, M., Kumari, P., Kundave, V.R., Singh, M., Banerjee, P.S., 2019. Immunoprophylactic evaluation of recombinant gametocyte 22 antigen of Eimeria tenella in broiler chickens. Parasitol. Res. 118. https://doi.org/10.1007/s00436-018-06198-2

Rafiqi, S.I., Garg, R., Reena, K.K., Ram, H., Singh, M., Banerjee, P.S., 2018. Immune response and protective efficacy of Eimeria tenella recombinant refractile body protein, EtSO7, in chickens. Vet. Parasitol. 258. https://doi.org/10.1016/j.vetpar.2018.06.013

Ramírez, J.D., Guhl, F., Messenger, L.A., Lewis, M.D., Montilla, M., Cucunuba, Z., Miles, M.A., Llewellyn, M.S., 2012. Contemporary cryptic sexuality in Trypanosoma cruzi. Mol. Ecol. 21. https://doi.org/10.1111/j.1365-294X.2012.05699.x

Rammensee, H.G., Bachmann, J., Emmerich, N.P.N., Bachor, O.A., Stevanović, S., 1999. SYFPEITHI: Database for MHC ligands and peptide motifs. Immunogenetics. https://doi.org/10.1007/s002510050595

Reid, A.J., Blake, D.P., Ansari, H.R., Billington, K., Browne, H.P., Bryant, J., Dunn, M., Hung, S.S., Kawahara, F., Miranda-Saavedra, D., Malas, T.B., Mourier, T., Naghra, H., Nair, M., Otto, T.D., Rawlings, N.D., Rivailler, P., Sanchez-Flores, A., Sanders, M., Subramaniam, C., Tay, Y.L., Woo, Y., Wu, X., Barrell, B., Dear, P.H., Doerig, C., Gruber, A., Ivens, A.C., Parkinson, J., Rajandream, M.A., Shirley, M.W., Wan, K.L., Berriman, M., Tomley, F.M., Pain, A., 2014. Genomic analysis of the causative agents of coccidiosis in domestic chickens. Genome Res. 24. https://doi.org/10.1101/gr.168955.113

Richard W. Gerhold, J., 2014. Gerhold, R. W. (2014). Overview of Coccidiosis in... - Google Scholar [WWW Document]. URL https://scholar.google.co.uk/scholar?hl=en&as_sdt=0%2C5&q=Gerhold%2C+R.+W.+%282014%29.+Overview+of+Coccidiosis+in+Poultry%3A+Coccidiosis%3A+Merck+Veterinary+Manual.&btnG=#d=gs_cit&u=%2Fscholar%3Fq%3Dinfo%3AiuEKVHVhrxQJ%3Ascholar.google.com%2F%26output%3Dc (accessed 6.19.21).

Rose, M.E., 1963. SOME ASPECTS OF IMMUNITY TO EIMERIA INFECTIONS. Ann. N.

Y. Acad. Sci. 113. https://doi.org/10.1111/j.1749-6632.1963.tb40677.x

Sanami, S., Zandi, M., Pourhossein, B., Mobini, G.R., Safaei, M., Abed, A., Arvejeh, P.M., Chermahini, F.A., Alizadeh, M., 2020. Design of a multi-epitope vaccine against SARS-CoV-2 using immunoinformatics approach. Int. J. Biol. Macromol. 164. https://doi.org/10.1016/j.ijbiomac.2020.07.117

Sanders, B., 2020. Global Animal Slaughter Statistics & Charts: 2020 Update - Faunalytics [WWW Document].

Sanders, B., 2018. Global Animal Slaughter Statistics And Charts - Faunalytics [WWW Document]. URL https://faunalytics.org/global-animal-slaughter-statistics-and-charts/ (accessed 6.29.21).

SAPA, 2017. SAPA Broiler Industry Summary on two pages 2017.

Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009. Genomic analysis of eimeria spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. J. Parasitol. 95. https://doi.org/10.1645/GE-1898.1

Seib, K.L., Zhao, X., Rappuoli, R., 2012. Developing vaccines in the era of genomics: A decade of reverse vaccinology. Clin. Microbiol. Infect. https://doi.org/10.1111/j.1469-0691.2012.03939.x

Shapiro, K., Bahia-Oliveira, L., Dixon, B., Dumètre, A., de Wit, L.A., VanWormer, E., Villena, I., 2019. Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food. Food Waterborne Parasitol. https://doi.org/10.1016/j.fawpar.2019.e00049

Sheykhsaran, E., Hemmat, N., Baghi, H.B., 2019. Influenza A virus and related secondary bacterial infections. Rev. Med. Microbiol. 30. https://doi.org/10.1097/MRM.0000000000000164

Shirley, M.W., Bedrník, P., 1997. Live attenuated vaccines against Avian coccidiosis: Success with precocious and egg-adapted lines of Eimeria. Parasitol. Today 13. https://doi.org/10.1016/S0169-4758(97)01153-8

Shirley, M.W., Smith, A.L., Tomley, F.M., 2005. The biology of avian Eimeria with an emphasis on their control by vaccination. Adv. Parasitol. https://doi.org/10.1016/S0065-308X(05)60005-X

Smith, A.L., Hesketh, P., Archer, A., Shirley, M.W., 2002. Antigenic diversity in Eimeria maxima and the influence of host genetics and immunization schedule on cross-protective immunity. Infect. Immun. 70. https://doi.org/10.1128/IAI.70.5.2472-2479.2002

Souillard, R., Le Maréchal, C., Ballan, V., Mahé, F., Chemaly, M., Le Bouquin, S., 2017. A bovine botulism outbreak associated with a suspected cross-contamination from a poultry farm. Vet. Microbiol. 208, 212–216. https://doi.org/10.1016/j.vetmic.2017.07.022

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry Coccidiosis: Design and Interpretation of Vaccine Studies. Front. Vet. Sci. https://doi.org/10.3389/fvets.2020.00101

Stanley, D., Wu, S.B., Rodgers, N., Swick, R.A., Moore, R.J., 2014. Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis

challenge model in chickens. PLoS One 9. https://doi.org/10.1371/journal.pone.0104739

Stucki, U., Braun, R., Roditi, I., 1993. Eimeria tenella: Characterization of a 5S ribosomal RNA repeat unit and its use as a species-specific probe. Exp. Parasitol. 76. https://doi.org/10.1006/expr.1993.1008

Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Dardé, M.L., Zhu, X.Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P., Sibley, L.D., 2012. Globally diverse Toxoplasma gondii isolates comprise six major clades originating from a small number of distinct ancestral lineages. Proc. Natl. Acad. Sci. U. S. A. 109. https://doi.org/10.1073/pnas.1203190109

Suprihati, E., Yunus, M., 2018. Evaluation of the antigenicity and immunogenicity of eimeria tenella by reproductive index and histopathological changes of cecal coccidiosis virulent live vaccine in broiler chickens. African J. Infect. Dis. 12. https://doi.org/10.2101/Ajid.12v1S.15

Tabarés, E., Ferguson, D., Clark, J., Soon, P.E., Wan, K.L., Tomley, F., 2004. Eimeria tenella sporozoites and merozoites differentially express glycosylphosphatidylinositol-anchored variant surface proteins. Mol. Biochem. Parasitol. 135. https://doi.org/10.1016/j.molbiopara.2004.01.013

Tahir Ul Qamar, M., Saleem, S., Ashfaq, U.A., Bari, A., Anwar, F., Alqahtani, S., 2019. Epitope-based peptide vaccine design and target site depiction against Middle East Respiratory Syndrome Coronavirus: An immune-informatics study. J. Transl. Med. 17. https://doi.org/10.1186/s12967-019-2116-8

Tallentire, C.W., Leinonen, I., Kyriazakis, I., 2016. Breeding for efficiency in the broiler chicken: A review. Agron. Sustain. Dev. https://doi.org/10.1007/s13593-016-0398-2

Tang, X., Liu, X., Yin, G., Suo, J., Tao, G., Zhang, S., Suo, X., 2018a. A novel vaccine delivery model of the apicomplexan Eimeria tenella expressing Eimeria maxima antigen protects chickens against infection of the two parasites. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.01982

Tang, X., Suo, J., Li, C., Du, M., Wang, C., Hu, D., Duan, C., Lyu, Y., Liu, X., Suo, X., 2018b. Transgenic Eimeria tenella expressing profilin of Eimeria maxima elicits enhanced protective immunity and alters gut microbiome of chickens. Infect. Immun. 86. https://doi.org/10.1128/IAI.00888-17

Terheggen, U., Drew, D.R., Hodder, A.N., Cross, N.J., Mugyenyi, C.K., Barry, A.E., Anders, R.F., Dutta, S., Osier, F.H.A., Elliott, S.R., Senn, N., Stanisic, D.I., Marsh, K., Siba, P.M., Mueller, I., Richards, J.S., Beeson, J.G., 2014. Limited antigenic diversity of Plasmodium falciparum apical membrane antigen 1 supports the development of effective multi-allele vaccines. BMC Med. 12. https://doi.org/10.1186/s12916-014-0183-5

The Poultry site, 2020. New report on the poultry and egg industries in South Africa | The Poultry Site [WWW Document]. URL https://www.thepoultrysite.com/news/2020/01/new-report-on-the-poultry-and-egg-industries-in-south-africa (accessed 6.29.21).

Tian, L., Li, W., Huang, X., Tian, D., Liu, J., Yang, X., Liu, L., Yan, R., Xu, L., Li, X., Song, X., 2017. Protective efficacy of coccidial common antigen glyceraldehyde 3-phosphate dehydrogenase (GAPDH) against challenge with three Eimeria species. Front.

Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01245

Tomley, F.M., Bumstead, J.M., Billington, K.J., Dunn, P.P.J., 1996. Molecular cloning and characterization of a novel acidic microneme protein (Etmic-2) from the apicomplexan protozoan parasite, Eimeria tenella. Mol. Biochem. Parasitol. 79. https://doi.org/10.1016/0166-6851(96)02662-X

Tomley, F.M., Clarke, L.E., Kawazoe, U., Dijkema, R., Kok, J.J., 1991. Sequence of the gene encoding an immunodominant microneme protein of Eimeria tenella. Mol. Biochem. Parasitol. 49. https://doi.org/10.1016/0166-6851(91)90071-D

Toseland, C.P., Clayton, D.J., McSparron, H., Hemsley, S.L., Blythe, M.J., Paine, K., Doytchinova, I.A., Guan, P., Hattotuwagama, C.K., Flower, D.R., 2005. AntiJen: a quantitative immunology database integrating functional, thermodynamic, kinetic, biophysical, and cellular data. Immunome Res. 1. https://doi.org/10.1186/1745-7580-1-4

Tsuji, N., Kawazu, S.I., Ohta, M., Kamio, T., Isobe, T., Shimura, K., Fujisaki, K., 1997. Discrimination of eight chicken Eimeria species using the two-step polymerase chain reaction. J. Parasitol. 83. https://doi.org/10.2307/3284302

Tyler, J.S., Treeck, M., Boothroyd, J.C., 2011. Focus on the ringleader: The role of AMA1 in apicomplexan invasion and replication. Trends Parasitol. https://doi.org/10.1016/j.pt.2011.04.002

Van Der Woude, M.W., Bäumler, A.J., 2004. Phase and antigenic variation in bacteria. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.17.3.581-611.2004

Venkatas, J., Adeleke, M.A., 2019. A review of Eimeria antigen identification for the development of novel anticoccidial vaccines. Parasitol. Res. https://doi.org/10.1007/s00436-019-06338-2

Wang, T., Sun, H., Zhang, J., Liu, Q., Wang, L., Chen, P., Wang, F., Li, H., Xiao, Y., Zhao, X., 2014. The establishment of Saccharomyces boulardii surface display system using a single expression vector. Fungal Genet. Biol. 64. https://doi.org/10.1016/j.fgb.2013.11.006

Wang, X. qiu, Wu, L. lin, Gao, Y., Zhang, Y., Weng, Y.B., Lin, R.Q., 2017. Evaluation of the protective effect of pVAX-EtMIC3-recombined plasmid against E. tenella in chicken. Parasitol. Res. 116. https://doi.org/10.1007/s00436-017-5383-4

Williams, R.B., Gobbi, L., 2002. Comparison of an attenuated anticoccidial vaccine and an anticoccidial drug programme in commercial broiler chickens in Italy. Avian Pathol. 31. https://doi.org/10.1080/03079450220136567a

Yan, M., Cui, X., Zhao, Q., Zhu, S., Huang, B., Wang, L., Zhao, H., Liu, G., Li, Z., Han, H., Dong, H., 2018. Molecular characterization and protective efficacy of the microneme 2 protein from Eimeria tenella. Parasite 25. https://doi.org/10.1051/parasite/2018061

Yang, G., Wang, C., Hao, F., Zhao, D., Zhang, Y., Li, Y., 2010. Studies on construction of a recombinant Eimeria tenella SO7 gene expressing Escherichia coli and its protective efficacy against homologous infection. Parasitol. Int. 59. https://doi.org/10.1016/j.parint.2010.06.010

Zhang, D.F., Xu, H., Sun, B.B., Li, J.Q., Zhou, Q.J., Zhang, H.L., Du, A.F., 2012. Adjuvant effect of ginsenoside-based nanoparticles (ginsomes) on the recombinant vaccine against Eimeria tenella in chickens. Parasitol. Res. 110. https://doi.org/10.1007/s00436-011-

2784-7

Zhang, G.L., Lin, H.H., Keskin, D.B., Reinherz, E.L., Brusic, V., 2011. Dana-Farber repository for machine learning in immunology. J. Immunol. Methods 374. https://doi.org/10.1016/j.jim.2011.07.007

Zhang, J., Chen, P., Sun, H., Liu, Q., Wang, L., Wang, T., Shi, W., Li, H., Xiao, Y., Wang, P., Wang, F., Zhao, X., 2014. Pichia pastoris expressed EtMic2 protein as a potential vaccine against chicken coccidiosis. Vet. Parasitol. 205. https://doi.org/10.1016/j.vetpar.2014.06.029

Zhang, J.R., Hardham, J.M., Barbour, A.G., Norris, S.J., 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell 89. https://doi.org/10.1016/S0092-8674(00)80206-8

Zhang, L., Li, X., Zhang, B., Meng, G., Xia, C., 2020. A Newly Recognized Pairing Mechanism of the a - and b -Chains of the Chicken Peptide–MHC Class II Complex. https://doi.org/10.4049/jimmunol.1901305

Zhang, T.E., Yin, L.T., Li, R.H., Wang, H.L., Meng, X.L., Yin, G.R., 2015. Protective immunity induced by peptides of AMA1, RON2 and RON4 containing T-and B-cell epitopes via an intranasal route against toxoplasmosis in mice. Parasites and Vectors 8. https://doi.org/10.1186/s13071-015-0636-5

Zhang, Z., Zhou, Z., Huang, J., Sun, X., Haseeb, M., Ahmed, S., Shah, M.A.A., Yan, R., Song, X., Xu, L., Li, X., 2020. Molecular characterization of a potential receptor of Eimeria acervulina microneme protein 3 from chicken duodenal epithelial cells. Parasite 27. https://doi.org/10.1051/parasite/2020014

Zhao, N., Ming, S., Lu, Y., Wang, F., Li, H., Zhang, X., Zhao, X., 2019. Identification and application of epitopes in EtMIC1 of eimeria tenella recognized by the monoclonal antibodies 1-A1 and 1-H2. Infect. Immun. 87. https://doi.org/10.1128/IAI.00596-19

Zhou, B. hua, Jia, L. shu, Guo, H. wei, Ding, H. yan, Yang, J. yun, Wang, H. wei, 2019. Eukaryotic elongation factor 2 is involved in the anticoccidial action of diclazuril in the second-generation merozoites of Eimeria tenella. Vet. Parasitol. 276. https://doi.org/10.1016/j.vetpar.2019.108991

Zhou, S., An, J., Zhang, P., Wang, L., 2012. Expession of microneme protein 4 from Eimeria tenella in the methylotrophic yeast Pichia pastoris, in: Proceedings - 2012 International Conference on Biomedical Engineering and Biotechnology, ICBEB 2012. https://doi.org/10.1109/iCBEB.2012.205

CHAPTER THREE

Genetic diversity of *Eimeria tenella* apical membrane antigen 1 (AMA1) from chickens in Mpumalanga province, South Africa

Abstract

Eimeria tenella is the primary cause of haemorrhagic coccidiosis in chicken. Understanding the genetic diversity of *Eimeria tenella* AMA1 (*Et*AMA1), the most crucial antigenic protein found in the various developmental stages of *Eimeria* parasites, is pivotal for vaccine improvement. Genetic diversity has been investigated in *Plasmodium* species and *Toxoplasma gondii*, but there is a paucity of information on *Eimeria* species. The current study aims to ascertain the prevalence of *Et*AMA1 and demonstrate the genetic diversity in this antigen. Four hundred fresh faecal samples were collected from 10 selected broiler chicken farms in Mpumalanga province from October 2018 to October 2019. The samples were examined under a compound microscope to confirm the presence of *Eimeria* oocysts. DNA was extracted from 200 samples containing *Eimeria* oocysts, and *E. tenella* was amplified using PCR-specific primers. Similarly, AMA1 was also amplified from the 103 isolates that were positive for *E. tenella* using PCR-specific primers. These PCR amplicons were sent to Inqaba biotech for sequencing and analyzed using MEGA6.06 and DnaSP programs. *Et*AMA1 sequences revealed a low level of diversity which was measured by nucleotide (0.0007) diversity, haplotype diversity (0.113) and haplotype number (3). Tajima's D test and Fu and Li's D* and F* tests provided strong evidence of significant balancing selection acting on *Et*AMA1 sequences. This study provides baseline information on *Et*AMA1, which can help design a regional vaccine against coccidiosis.

Keywords: Apical membrane antigen 1; chicken; *Eimeria tenella*; Antigenic diversity; Epitope, Vaccine

3.0 Introduction

South Africa is one of the African countries, which produces poultry and its processed products. The poultry sector contributes about R5 171 million to the countries' economy every year (Bolton, 2015). Broiler chickens are produced in all South African provinces, North West, Western and Northern Cape, Mpumalanga and KwaZulu– Natal Provinces were the largest producers of broiler meat in South Africa in 2012 (DAFF, 2013). In 2012, Mpumalanga Province was the third-largest producer of broiler meat in the whole of South Africa. The province accounted for 21% of the total broiler meat produced in South Africa (DAFF, 2013). However, these animals are susceptible to parasitic infections, which reduces productivity and leads to economic loss (Bolton, 2015).

Chicken coccidiosis is one of the most economically parasitic diseases facing the poultry industry (Blake et al., 2020). This disease which is caused by *Eimeria* protozoan parasites falls under the apicomplexan phylum. The disease results in a significant economic loss (Gharekhani et al., 2014). Its impact is recognized worldwide due to its effect on the agricultural sector (Bachaya et al., 2012). There are currently seven *Eimeria* species known to cause coccidiosis in chicken namely; *E. acervulina*, *E. praecox*, *E. mitis*, *E. tenella*, *E. necatrix*, *E. brunetti*, and *E. maxima* (Gharekhani et al., 2014). Among these species, *Eimeria tenella* is the most pathogenic and causes body weight loss, haemorrhagic diarrhoea, severe lesion of caeca and eventually death (Witcombe and Smith, 2014). Therefore, this species is best deliberated on and therefore was designated as the choice species in this study.

The current control measure against poultry coccidiosis is dominated by anticoccidial drugs or vaccination with either live or attenuated species of *Eimeria* (Soutter et al., 2020). The challenge with anticoccidial drugs in the control of coccidiosis is associated with parasites resistance (Lawal et al., 2016). Live anticoccidial vaccines are an efficient alternative to anticoccidial drugs as they stimulate durable protective immune protection (Soutter et al., 2020). In some regions, live attenuated parasite as an alternative measure has become popular thus far (Peek and Landman, 2011). However, the vaccine's uptake remains limited by high production cost (Eckford et al., 2013; Soutter et al., 2020). Hence there is an urgent need for the development of cost-effective subunit vaccines to contain chicken coccidiosis.

Several antigenic agents have been characterized as potential vaccine candi3dates (Suprihati and Yunus, 2018) using different strategies (Kundu et al., 2017; Rafiqi et al., 2019). *E. tenella* antigenic proteins that have been identified to date include, but not limited to

apical membrane antigen 1 (AMA1) (Li et al., 2018; Yan et al., 2018), ubiquitin-conjugating enzyme (UCE) (Liu et al., 2017), Glyceraldehyde 3-Phosphate Dehydrogenase (Tian et al., 2017) and microneme-3 protein (MIC3) (Huang et al., 2018).

Han *et al*., 2016 demonstrated that *Et*AMA1 has high immune production and is described as a leading potential vaccine candidate (Han et al., 2016). However, the antigenic diversity and population structure of AMA1 in closely related apicomplexans such as *Plasmodium* have hindered vaccine development (Blake et al., 2015). As a result, an in-depth understanding of antigenic diversity in the parasite population is essential for designing a successful design against coccidiosis. Presently, there is inadequate understanding of the genetic diversity of AMA1 in the *Eimeria* parasite. To the best of our knowledge, no study has been reported in South Africa on the genetic diversity of *Et*AMA1. Hence, the current study aims to address these gaps and advance the development of an effective AMA1 based vaccine.

3.1 Materials and Methods

3.1.1 Ethical statement

Ethical clearance was approved by the animal research ethics committee (AREC) at the University of Kwa-Zulu Natal under the project licence: AREC/063/018M. All animal studies and sampling protocols applied here were followed as per AREC guidelines and regulations.

3.1.2 Study area

Faecal samples for this study were collected from Mpumalanga Province, South Africa, within latitude 26° 0′ 0″ S and longitude 30° 0′ 0″ E. The province is the second-smallest province with a surface area of 76 495 km^2 and 4 592 187 estimated population size (Stats-SA, 2020). Mpumalanga is located in the north-eastern region of South Africa. This province consists of three district municipalities: Ehlanzeni, Gert Sibande and Nkangala, which are further divided into 17 local municipalities. For this study, 8 of the 17 local municipalities of Mpumalanga province were randomly and conveniently selected for sampling (Fig. 1). A list of farms was obtained from the internet, and all the contacted farms participated in the study. Mpumalanga province is one of the largest producers of chickens in South Africa.

A recent survey on the regional distribution of layers chickens in South Africa showed that a total of 2 890 851, representing 9.5% of the total layers chickens reared in South Africa in 2019, were from Mpumalanga province (SAPA, 2019). Also, Mpumalanga province was the second-largest producer of broiler chickens in South Africa in 2019 (SAPA, 2019). A total of 24, 053, 556, representing 20.9 of the total broiler meats produced in South Africa in 2019, were from Mpumalanga province (SAPA, 2019).

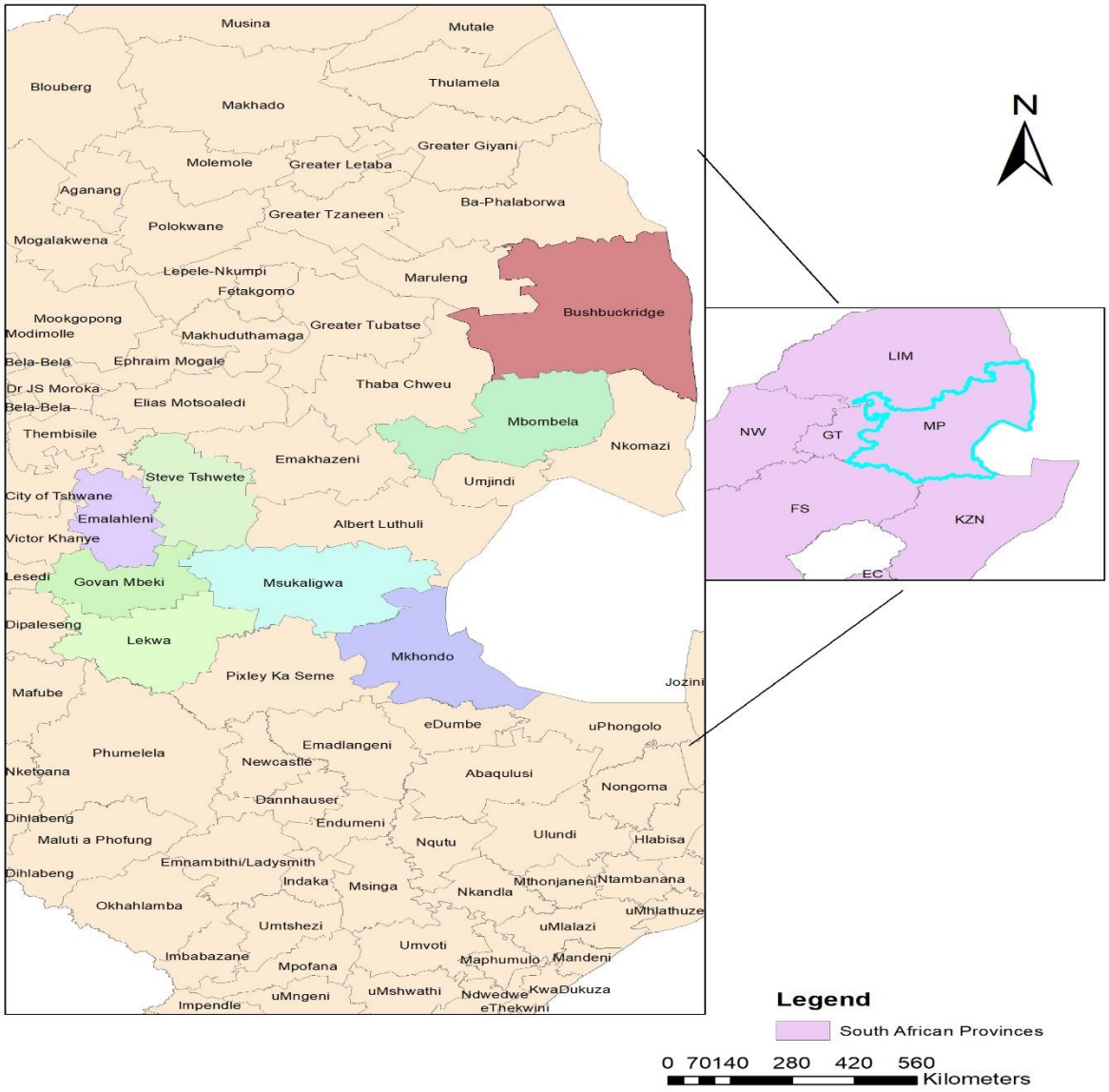

Fig. 1: Geographic distribution of study sites selected. The samples were collected in 8 local municipalities of Mpumalanga province, i.e. Bushbuckridge, Mbombela, Steve Tshwete, Emalahleni, Govern Mbheki, Lekwa, Msukalingwa and Mkhondo.

3.1.3 Sampling

A total of 400 fresh faecal droppings were collected from 10 selected farms (Shown in a supplementary information). Farm location 'D2' has the highest numbers of chickens (1500), followed by sites C, D1, A, E, I, F, G, J, and H, with 1100, 1000, 850 and 800, 251, 121, 91, 87 and 83 broiler chickens respectively. During sampling, some of the chickens reared in back yards showed clinical sign which was diarrhoea, with watery and dark faeces. A total of 52 samples each were collected from farms C and D1, 44 samples were collected from farm A, 40 samples each were collected from sites E, F, G, and I, 36 samples from farm B, 20 samples each from locations H and J, and 16 samples from farm D2. All the farms were visited between October 2018 and October 2019. During the time of sampling, the age of chickens ranged from 2 to 8 weeks. Samples were collected in 50 ml falcon tubes prefilled with 20 ml of 2% (w/v) potassium dichromate (K2Cr2O7) following a "W" (wall to wall) pathway as previously described by Kumar *et al*., 2014, labelled and transported to the Genetics laboratory at the University of KwaZulu-Natal and refrigerated at 4 ℃ until further use. This was done to preserve the oocyst integrity (Duszynski and Wilber, 1997).

3.1.4 Sample size

To ensure statistical power and reliability of results, the sample size was calculated based on the formula described by Thrusfield, 1997.

$N = Z2pq /d2$

Where N = sample size

Z = appropriate value for the standard normal deviate for the desired confidence = 1.96

p = prevalence

q = 1 – p

d = level of significance (0.05)

The occurrence of coccidiosis in Mpumalanga is not known, thus the sample size was determined using 50% prevalence.

$N = 1.962 \times (0.50 \times 0.50)$

$(0.05)2$

$= 384.16$

3.1.5 Sample processing and Microscopic examination

All collected samples were screened for the presence of *Eimeria* oocyst following a protocol described by Kumar *et al*., 2014 with the following magnifications. First, Falcon tubes with faecal samples were vortexed for 30 seconds. Following this, about four scoops of the faecal sample were transferred into a glass beaker, soaked in 40 ml of distilled water, and homogenized by stirring using a scoop. The solution was then sieved through a metal sieve into a 50 ml falcon tube; this was repeated for all 400 samples. Lastly, the falcon tubes were filled with a saturated salt solution to allow the oocyst to float and covered with a coverslip and left for 10 minutes. The samples were examined under the microscope (OMAX-microscope) at 400x magnification to confirm the presence of unsporulated *Eimeria* oocysts. Photomicrographs of *Eimeria* oocysts were captured using a digital camera connected to the microscope. Two hundred (200) samples that were positive *for Eimeria* oocyst were further used for DNA extraction.

3.1.6 Extraction and purification of Genomic DNA

Genomic DNA was extracted from the samples with positive oocyst using a Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (ZYMO Research, USA) as per the manufacturer's instructions. The quality and concentration of the extracted DNA was measured using a Thermo Scientific TM NanoDrop 2000 spectrophotometer (ThermoFisher Scientific Inc, USA) at the absorbance of 260nm. The extracted genomic DNA was then stored at -20°C till further use.

3.1.7 Polymerase chain reaction (PCR)

3.1.7.1 Amplification of *Eimeria tenella* ITS-1 gene

The extracted DNA from 200 samples was first screened for *E. tenella* species by targeting ITS-1 gene using species-specific primers (5'-3'): AATTTAGTCCATCGCAACCCT and CGAGCGCTCTGCATACGACA as described by Lew et al. (2003). The amplification was carried out in 25 µl reaction volume containing 5 µl of sample DNA, 1 µl of each forward and reverse primer (10 µM of stock solution), 12.5 µl of dream Taq and 5.5 µl ddH2O. Cycling conditions comprised of a pre-denaturation step at 94°C for 3 min, followed by 30 cycles with 30sec of denaturation at 94°C, annealing temperature was set at 65°C for 30sec, extension, 72°C for 30 sec and final extension of 15 min at 72°C. Out of these, 136 were positive for *E. tenella*.

3.1.7.2 *Eimeria tenella* AMA1 amplification

Amplification of AMA1 was done on 136 samples positive for *E. tenella* using newly synthesized *Et*AMA1 primers (5'-3'): ACCAGCCCACATCTCAAGAC and GGATTGAACACCTTGCTGCT 3'). Amplification was carried out in 25 µl reaction volume containing 5 µl of sample DNA, 1 µl of each forward and reverse primer (10 µM of stock solution) , 12.5 µl of dream Taq and 5.5 µl ddH2O. Cycling conditions were as follows: Pre-denaturation at 94 °C for 3 min, denaturation, 94 °C for 30 sec, annealing, 59.5 °C for 30 sec, extension, 72 °C for 90 sec and a final extension at 72 °C for 7 min. Thus, out of 136 samples positive for *E. tenella,* AMA1 was amplified in only 103 samples.

3.1.7.3 Gel visualization

One hundred and three (103) *Et*AMA1 PCR products were visualized by electrophoresis on 1.5% (w/v) agarose gels stained with 2 µl ethidium bromide at 100 V for 30 min. Negative control was used for each set of primer, including all other reagents but short of target DNA. Out of 103 *Et*AMA1 amplicons obtained, only 50 with brighter/clear bands were selected for further analysis.

3.1.7.4 Sequencing of PCR products, alignment and phylogenetic analysis

The amplified *Et*AMA1 PCR amplicons (n =50) were sent to Inqaba Biotec in Pretoria, South Africa, for Sanger sequencing. Out of 50 amplicons sent to Inqaba Biotech, only 35 were used for further analysis, with the band length ranging from 190 – 245pb. Sequences were edited, trimmed, and consensus sequences of 190bp were generated using MEGA6.06 (Tamura et al., 2013). The sequences in the current study were then compared with *Et*AMA1 sequences in the GenBank using the BLASTn tool. A total of 56 *Et*AMA1 sequences with 100% identity to the 35 *Et*AMA1 sequences obtained from this study were retrieved and aligned using clustalW in MEGA6.06 (Tamura et al., 2013). A phylogenetic tree was then constructed following a maximum likelihood method with 1000 bootstrap.

3.1.7.5 *Et*AMA1 sequence diversity, haplotype analyses and natural selection

Genetic diversity was determined using DnaSP software ver. 5 (Librado and Rozas, 2009). The obtained data comprised of the mean number of nucleotide differences (K), nucleotide diversity (π) (calculated using the Jukes-Cantor correction and sliding window method of 100 bases with a step size of 25bp), the total number of haplotypes (*H*), haplotype diversity (*Hd*) and their corresponding standard deviation.

Furthermore, the numbers of the synonymous (*dS*) to nonsynonymous mutations (*dN*) were obtained and compared with a Z-test in MEGA ver. 6.06 following Nei and Gojobori's method (Nei and Gojoborit, 1986). Tajima's (D) test as well as Fu and Li′s (D*) and Fu and Li′s (F*) tests were calculated based on the number of mutations and sliding window of 100bp with 25-bp step size to test the neutral theory of evolution using DnaSP software. The *Et*AMA1 sequences in this study and the sequences retrieved from GenBank were further subjected to NETWORK software ver, 5.0 (http://www.fluxus-engineering.com) to generate the relationship among the haplotypes following a median joining approach.

3.2 Results

3.2.1 Microscopic examination of *Eimeria* oocysts from chicken droplet from Mpumalanga, South Africa.

Of the 400 samples examined microscopically, *Eimeria* oocysts (Fig. 2) were observed in 200 (50%) samples (Table 1). In all, 29 (72.5%) of the samples collected from farm E were positive for *Eimeria* oocysts, 28 (70%), 35 (67.3%), 27 (61.4%), 26 (50%), 20 (50%) of samples collected from sites I, C, A, D1 and G also harbour *Eimeria* oocysts respectively, while none was detected in all the samples obtained from farm D2 (Table 1).

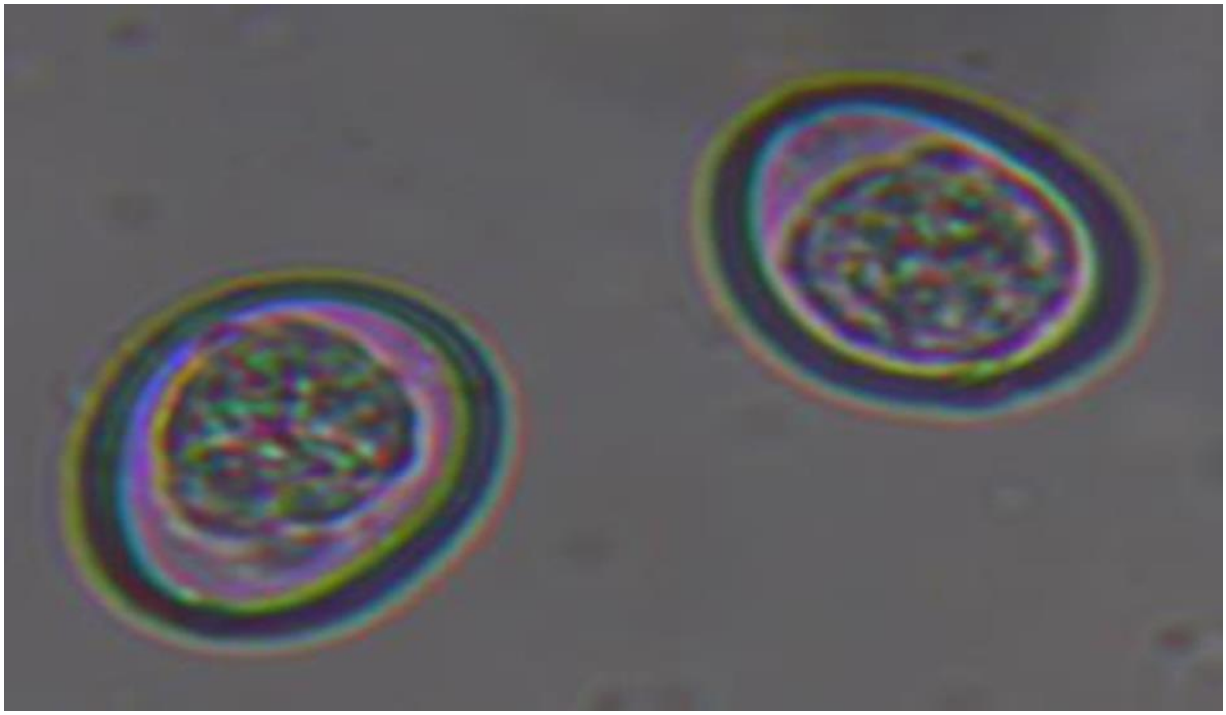

Fig. 2: Microscopic examination of unsporulated *Eimeria* oocysts observed under a 40X objective lens.

Table 1: Occurrence of *Eimeria* oocysts in different farms in Mpumalanga, South Africa.

Farms	Age (Weeks)	Population size (N)	No. of samples collected	Sample positive for *Eimeria* oocyst	Occurrence (%)
A	7	850	44	27	61,4
B	8	107	36	13	36,1
C	4	1100	52	35	67,3
D1	6	1000	52	26	50,0
D2	2	1500	16	0	0,0
E	5	800	40	29	72,5
F	6	121	40	17	42,5
G	6	91	40	20	50,0
H	5	83	20	3	15,0
I	4	251	40	28	70,0
J	6	87	20	2	10,0
Total		**5990**	**400**	**200**	**50.0**

3.2.2 PCR amplification of *Eimeria tenella* from broiler chickens in Mpumalanga province of South Africa.

A total of 136 (68%) of the samples were positive for *E. tenella* (Fig. 3). The incidence of *E. tenella* was low in most of the farms visited, except at sites 'I', E, C, and F, where the prevalent rates were 28 (70%), 20 (50%), 19 (47.5%), 17 (42.5%) respectively, and none of the samples collected from farm 'D2' had *E. tenella* (Fig. 4).

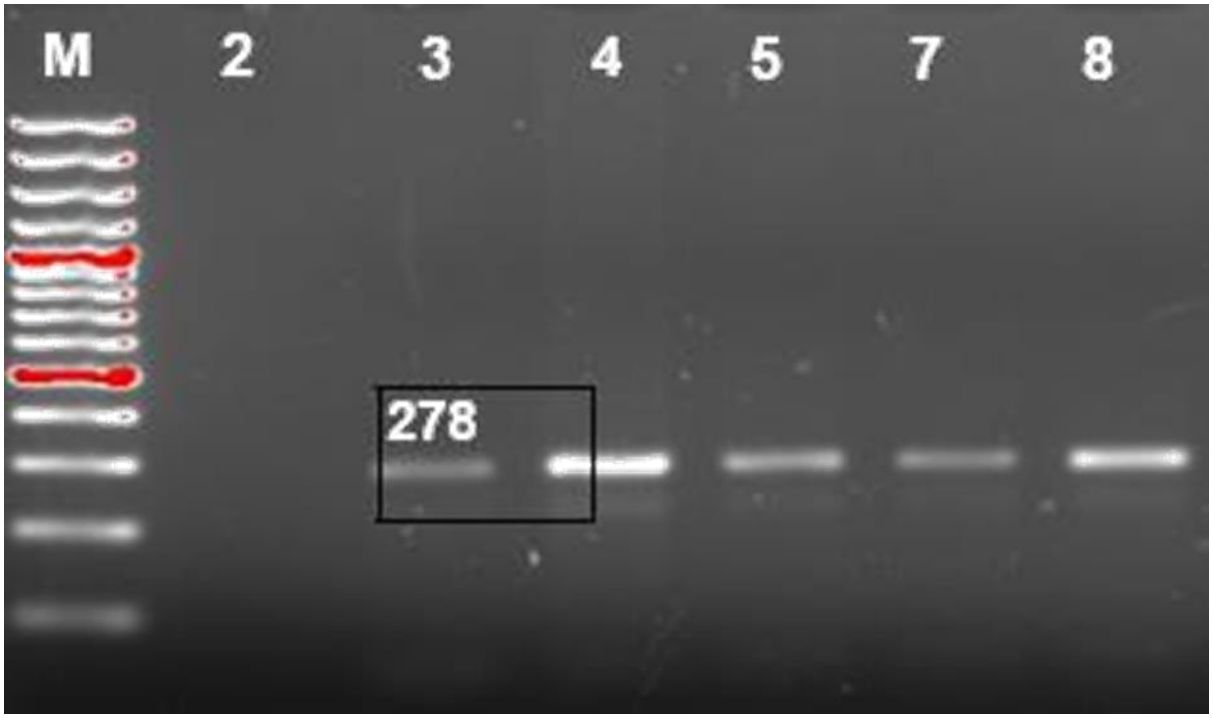

Fig. 3: PCR amplification of *Eimeria tenella*. M: 100bp plus Molecular weight marker; Lane 2: negative control; Lane 3-8: 278bp positive samples.

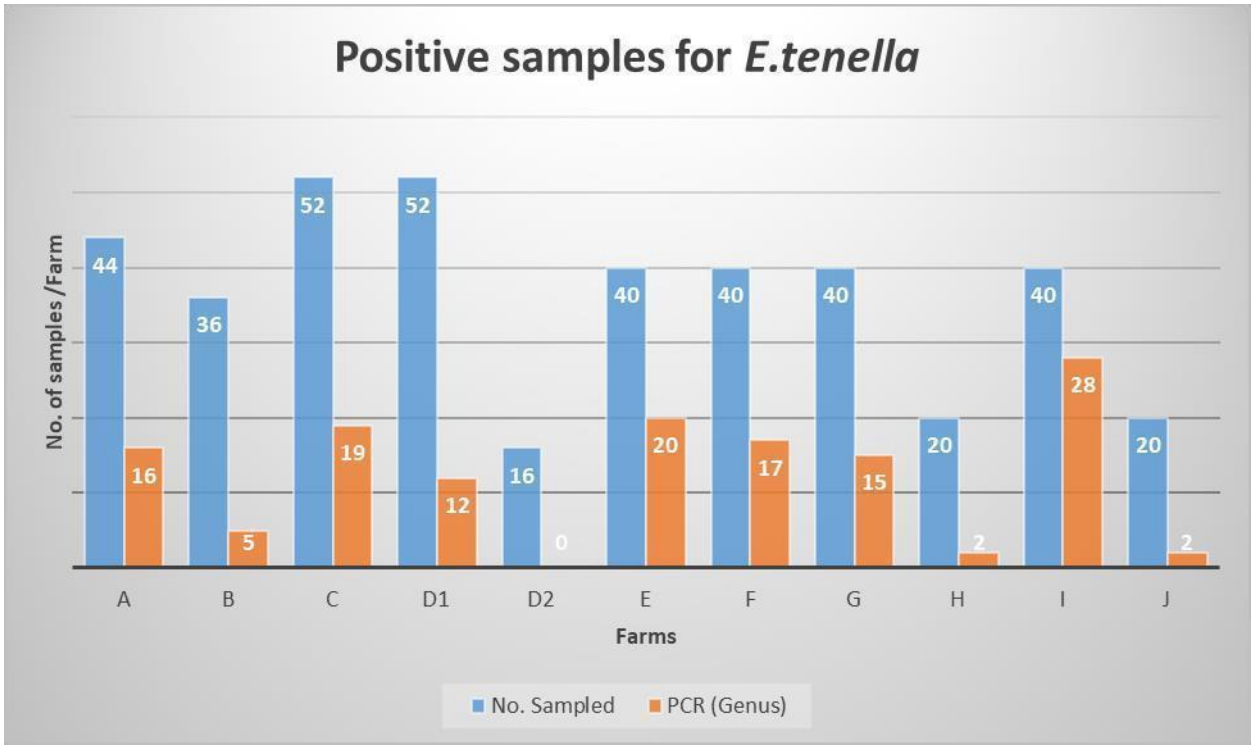

Fig. 4: Occurrence of *Eimeria tenella* in different farms around Mpumalanga province.

3.2.3 Incidence and prevalence of *Eimeria tenella* Apical Membrane Antigen 1

*Et*AMA1 was present in 103 (75.7%) of the *E. tenella* (Fig.5). The highest prevalence of 52.5% was detected for *Et*AMA1 in the samples obtained from farm I, followed by farms E, F, G, A, C, D1, and B with prevalence rates of 17 (42.5%), 14 (35.0%), 12 (30.0%), 12 (27.3%), 13 (25.0%), 9 (17.3%), and 5 (13.9%) respectively while none was detected from samples obtained from D2, H and J respectively (Table 2).

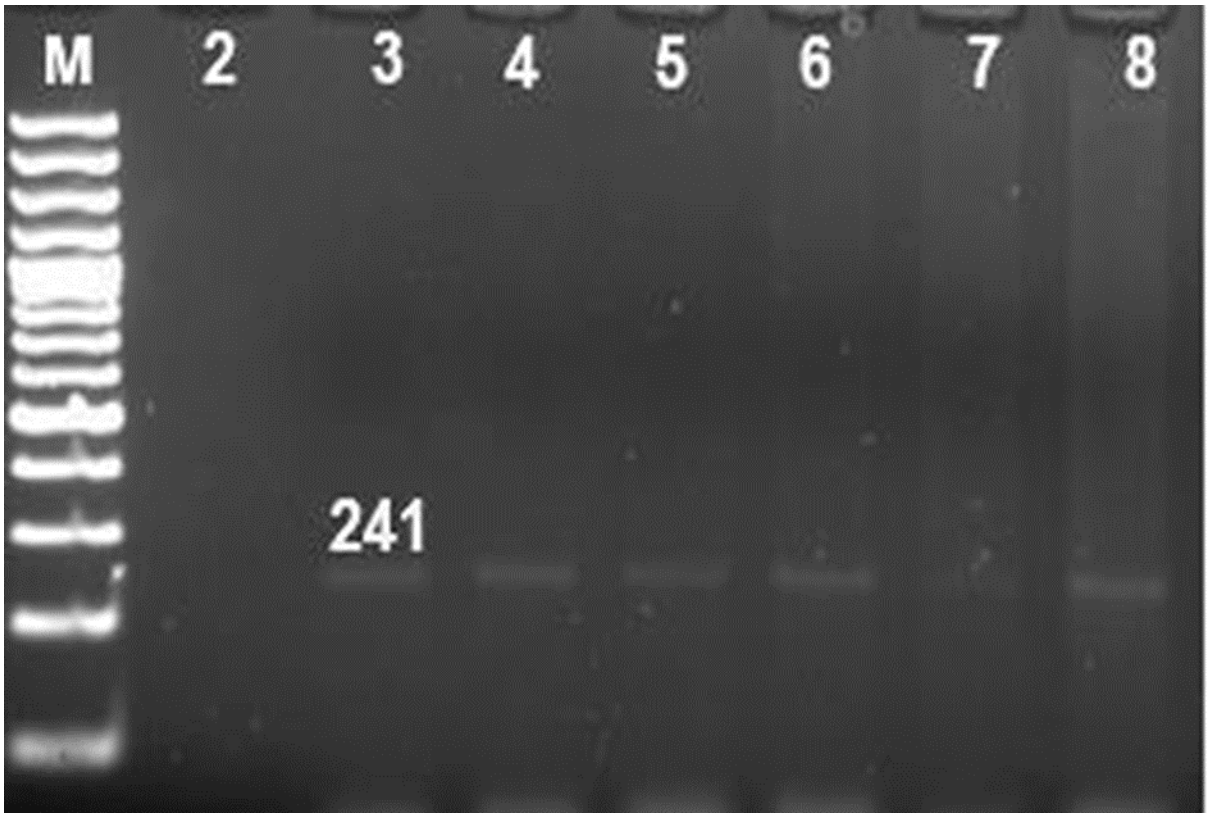

Fig. 5: PCR amplification of *Et*AMA1. M: 100bp plus Molecular weight marker; Lane 2: negative control; Lane 3-8: 241bp positive samples.

Table 2: Occurrence and Prevalence of *Et*AMA1 from different poultry farms in Mpumalanga, South Africa.

Farms	**No. of samples**	**Samples positive for *E. tenella***	**Samples positive for AMA1**	**Prevalence of AMA1 (%)**
A	44	16	12	27,3
B	36	05	5	13,9
C	52	19	13	25,0
D1	52	12	9	17,3
D2	16	0	0	0,0
E	40	20	17	42,5
F	40	17	14	35,0
G	40	15	12	30,0
H	20	02	0	0,0
I	40	28	21	52,5
J	20	02	0	0,0
Total	**400**	**136**	**103**	**75.7**

3.2.4 Genetic diversity among *Et*AMA1 sequences from Mpumalanga province.

In this study, the average number of nucleotide differences (k) was estimated to be 0.114. Therefore, the total nucleotide diversity (π) and haplotype diversity (*Hd*) were 0.0007±0.0005 and 0.113±0.072, respectively, as shown in Table 3. Nucleotide diversity analysis using a sliding window method ranged from 0 to 0.0014. The nucleotide position 149 had the highest genetic diversity of 0.0014 (Table 4).

Table 3: Genetic diversity among *Et*AMA1 sequences from Mpumalanga province.

Antigen	**N**	**S**	**k**	***H***	**π±S.D**	***Hd*±S.D**
*Et*AMA1	35	2	0.114	3	0.0007±0.0005	0.113±0.072

N: number of sequences used; S: number of polymorphic sites; k: average number of nucleotide differences; *H*: number of haplotypes; π: nucleotide diversity based on jukes cantor; *Hd*: haplotype diversity

Table 4: Sliding window showing results of nucleotide diversity among Mpumalanga *Et*AMA1 sequences.

Window	Midpoint	π
1-132	82	0.0000
58-157	107	0.0006
83-182	132	0.0006
108-190	149	0.0014

3.2.5 Comparative genetic diversity between *Et*AMA1 sequences from this study and those retrieved from GenBank.

As shown in Table 5, comparative genetic diversity between *Et*AMA1 sequences from Mpumalanga province in this study and those retrieved from GenBank resulted in 87 segregation sites. The overall nucleotide diversity (π) and haplotype diversity (*Hd*) were 0.471±0.014 and 0.495±0.031, respectively. The average numbers of the synonymous (dS) and nonsynonymous mutations (dN) mutations were 0.669 and 0.573, respectively, and the ratio was ($P<0.05$ in Z-test). Furthermore, Tajima's D test (4.560; $P<0.001$), Fu and Li′s D* (2.072; $P < 0.02$) and Fu and Li′s F* (3.724; $P<0.02$) showed significant balancing selection indicated by positive values. However, further analysis for neutrality tests using the sliding window method zooming in specific regions showed that all three tests detected significant positive values, as shown in Table 6.

Table 5: Comparative genetic diversity between *Et*AMA1 sequences from this study and those retrieved from Genbank.

Antigen	**N**	**S**	**π ±S.D**	**K**	***H***	***Hd*±S.D**	**dN/dS**	**D**	**D***	**F***
*Et*AMA1	91	87	0.471± 0.014	41.193	4	0.495± 0.031	0.856	4.560	2.072	3.724

N: number of sequences used; S: number of polymorphic sites; π: nucleotide diversity based on jukes cantor; k: average number of nucleotide differences; *H*: number of haplotypes; Hd: haplotype diversity; dN/dS: ratio of nonsynonymous to synonymous mutations; D: Tajima's, Fu and Li's D* and F* tests.

Table 6: Sliding window showing Tajima's D and Fu and Li's D* and Fu and Li′s F* of *Et*AMA1 sequences from this study and those retrieved from Genbank.

Window	Midpoint	Tajima's D	D*	F*
1-132	79	4.627***	2.186**	3.804**
55-157	104	4.479***	1.977**	3.581**
80-183	132	4.471***	1.766**	3.568**
105-190	148	4.186***	1.582*	3.133**

***P<0.01, **P<0.02, *P<0.05

3.2.6 Phylogenetic tree and Haplotype network analysis.

A total of 91 *Et*AMA1 combined sequences were used to construct a phylogenetic tree and haplotype network. The 35 *Et*AMA1 sequences were from this study, and 56 *Et*AMA1 reference sequences were from Nigeria, UK Houghton, Venezuela, Libya, India, China, Egypt, Germany, Japan, UK Weybridge, and the US. The maximum likelihood tree (Fig. 6) resulted in two major clusters. The sequences from this study were clustered together, and they are diverse compared to the GenBank sequences, and they seem to have evolved recently since they have more than two clades after their first evolution. The results from the phylogenetic tree were supported by the haplotype network shown in Fig. 7. There are four haplotypes`identified; Haplotype 1-3 belongs to Mpumalanga, while Haplotype 4 contains sequences from different countries.

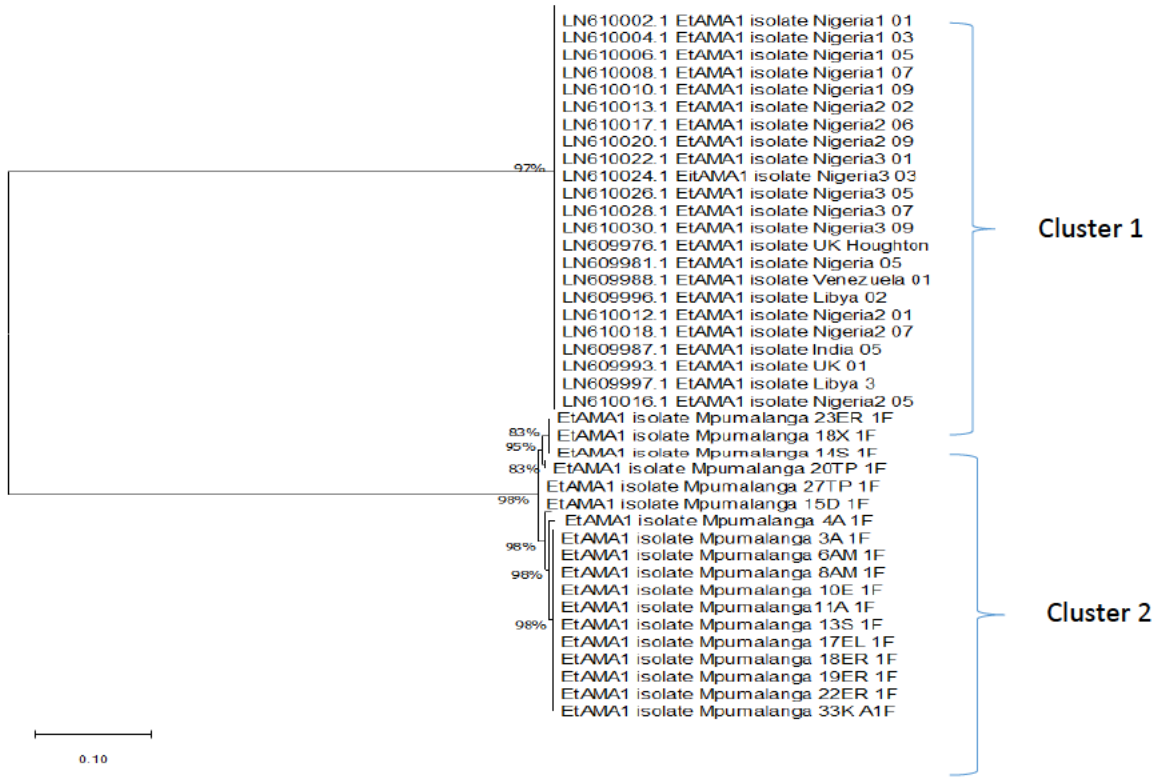

Fig. 6: Maximum likelihood tree based on *Et*AMA1 sequences from this study and those retrieved from GenBank.

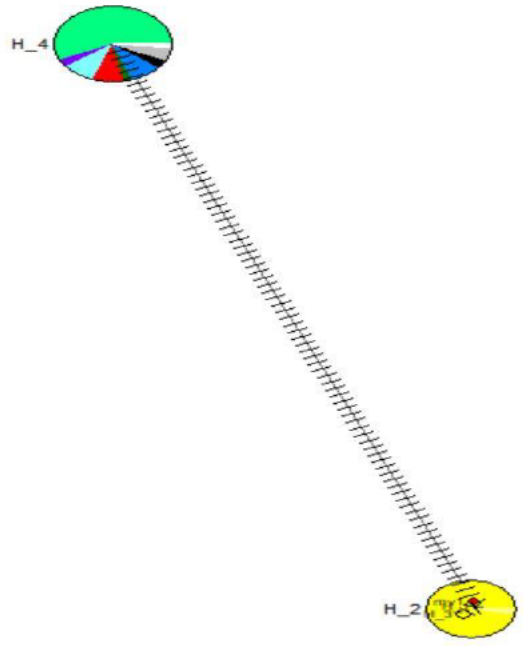

Fig. 7: Median-joining network representing the relationship between the *Et*AMA1 sequences from this study and those retrieved from GenBank. Yellow: Mpumalanga South Africa; Green: Nigeria; Grey: UK; Black: Germany; Red: India; Light blue: Libya; Purple: Egypt; Deep blue: Venezuela; Army green: China; White: Japan.

3.3 Discussion

Coccidiosis, caused by *Eimeria* species, is a common parasitic infection affecting livestock (Kvičerová and Hypša, 2013) but more common among broiler chickens (Borgonovo et al., 2020). The disease reduces animal performance and welfare, resulting in high mortality (Abdelli et al., 2020). Chicken coccidiosis is currently controlled by anticoccidial drugs and/or live parasite vaccination (Soutter et al., 2020). However, the production of cost-effective recombinant anticoccidial vaccines against this pathogen is a vital goal to control coccidiosis. Under the microscopic analysis, it was observed that 50% of the samples were infected, which was evident by the presence of unsporulated oocysts.

To overcome the confounding limitations of morphological findings, it was imperative to incorporate molecular diagnostics using the PCR technique. The genes marker ITS1 and ITS2 rDNA repeat are primarily used in PCR-based analysis of the occurrences and prevalence of *Eimeria* spp (Kumar et al., 2014). The ITS1 and ITS2 rDNA repeat sequences have also been used to classify genetically definite genotypes in different geographical areas and farms and establish the relationship between disease and performance (Schwarz et al., 2009). In addition, the ITS regions are utilized in molecular and phylogenetic analyses due to their sensitivity and specificity (Jiang et al., 2012). Using the ITS1 sequence, *E. tenella* was detected in 136 (68%) samples.

The 68% prevalence of *E. tenella* species in broiler chicken farms at Mpumalanga province of South Africa is in accordance with the recently reported 68.4% prevalence of *E. tenella* found in the KwaZulu-Natal province of South Africa (Fatoba et al., 2020). This buttress the efficacy of species-specific primers for the detection of different species of *Eimeria*. The absence of *E. tenella* on-farm D2 is in agreement with another report (Schwarz et al., 2009) and could be attributed to the level of hygiene on farms (Debbou-Iouknane et al., 2018).

Apical membrane antigen 1 domicile in an invasive zoites has been an essential protein in *E. tenella* invasion (Tyler et al., 2011). AMA1 is very important in the formation and maintenance of adhesion between parasites and hosts cells membranes (moving junction), through which the parasites move (glide) into the intracellular vacuole (Tyler et al., 2011; Pastor-Fernández et al., 2018). AMA1 has been identified as one of the antigens providing optimal levels of immunoprotection and, therefore, may be applicable as a suitable candidate for vaccine development.

Eimeria tenella AMA1 at different developmental stages has been identified using both qRT-PCR and Western Blotting, and it was observed that *Et*AMA1 was expressed at higher levels in sporozoites than in the other developmental stages (Jiang et al., 2012). The current study utilizes primers specific PCR targeting *Et*AMA1 sequences to screen *E. tenella* positive samples obtained from different farms in the Mpumalanga province of South Africa and found a 75.7% prevalence of *Et*AMA1 in that province.

The prevalent rate of *Et*AMA1 in this study is lower than the previous report (Tomley, 1997). AMA1 antigen as a potent vaccine candidate for *Eimeria* can also be pertinent for *Neurospora caninum*, *T. gondii*, and several *Plasmodium* species (Jiang et al., 2012). Previous studies have shown that *Et*AMA1 binding peptides inhibited the invasion of host cells by *E. tenella* sporozoites (Ma et al., 2019). *Et*AMA1 has also been demonstrated to offer partial protection against heterologous infection with *E. maxima* when the AMA1 protein from *E. maxima* was expressed as a live vectored vaccine and also partially protect the host cells against homologous challenge with *E. tenella* when administered as a recombinant protein vaccine (Pastor-Fernández et al., 2018).

Evaluating genetic variation assists in the identification of potential antigens crucial for vaccine development. High polymorphism exists among several *Plasmodium vivax* and *Plasmodium falciparum* potential antigens pause a serious problem in the effectiveness of vaccines (Chenet et al., 2008). Hence the current study determined the genetic diversity that exists among AMA1 vaccine candidate in *E. tenella* . The genetic diversity among *Et*AMA1 sequences in this current study was determined by evaluating the nucleotide diversity (0.0007), the number of haplotypes (3) as well as haplotype diversity (0.113). The number of haplotypes revealed in this study is equal to those reported from United States (3 haplotypes in 6 isolates, Blake et al., 2015). Mpumalanga *Et*AMA1 sequences revealed a low level of diversity. However, when compared with the sequences from GenBank, diversity parameters were slightly higher. The results from the current study are in accordance with the previous report (Blake et al., 2015) and favour the use of *Et*AMA1 in future subunit vaccine development (Blake et al., 2015).

The low genetic diversity observed among *Et*AMA1 sequences was supported by the phylogenetic tree and haplotype network. The phylogenetic analysis classified *Et*AMA1 sequences into cluster 1 and 2, and sequences clustered together are known to have a close relationship with each other. Mpumalanga sequences were clustered together with strong support ranging from 83% to 98%.

The *Et*AMA1 sequences from this study formed two major clades, which further formed sister clades. The phylogenetic analysis shows little or no diversity between Mpumalanga sequences, which was expected since the samples were collected within the province. Haplotype network revealed 4 haplotypes, 3 of which consist of samples from Mpumalanga. Haplotype network revealed that Mpumalanga sequences are closely related to each other and distantly related to sequences from different countries (Table S1), where matching sequences are presented by nodes.

The ratio of nonsynonymous to synonymous mutations (dN/dS) among *Et*AMA1 coding sequences showed that the number of nonsynonymous mutations is less than the number of synonymous mutations. The obtained ratio (dN/dS) in this study is less than one (0.856), indicating that negative/purifying Darwinian selection plays a most crucial role in the diversity observed among the *Et*AMA1 sequences of this study and *Et*AMA1 sequences retrieved from GenBank. Purifying or negative selection can be described as removing the unfit within the population (Brunet et al., 2021). Traditionally, the ratio of nonsynonymous to synonymous mutations must be significantly greater than 1 to show a positive Darwinian selection model of neutral evolution and purifying selection must be rejected (Nei and Gojoborit, 1986)). The results of dN/dS obtained in this study are not in agreement with the neutrality tests denoted by Tajima's and Fu and Li's tests. A broader survey on analysis of a selection of signatures conducted by Reid *et al*., 2014 also indicated purifying/negative selection with nonsynonymous to synonymous (Ka/Ks) ratio of 0.2 (Reid et al., 2014). Similar results by Blake *et al*., 2015 indicated purifying/negative selection while significant positive values were obtained for neutrality tests at domain II with the ratio of 0.823 (Blake et al., 2015). Moreover, the ratio of nonsynonymous to synonymous mutations may not be a helpful discriminator for identifying candidate vaccine antigens (Blake et al., 2015).

Tajima's D and Fu and Li's D* and F* neutrality tests were used to evaluate the occurrence and extent of departure from neutral evolution among the *Et*AMA1 sequences in this study and those obtained from GenBank. The results of these tests showed significant evidence of balancing selection denoted by positive values. Similar results of significant balancing selection were observed among AMA1 of closely related Apicomplexa parasites including, *Plasmodium* (Mehrizi et al., 2013; Lumkul et al., 2018; Wang et al., 2019). However, Blake *et al*., 2015 reported contrasting results of neutrality tests, which detected no significant signatures of balancing selection (Blake et al., 2015).

This opposing result could be because Blake *et al*., 2015 use the entire length of *Et*AMA1 sequences compared to the partial *Et*AMA1 length used in the current study.

3.4 Conclusion

In conclusion, the prevalence of *E. tenella* in most of the chicken droplets obtained from different farms in Mpumalanga province and those recently reported from other South African provinces showed that subclinical coccidiosis infection caused by *E. tenella* might be common among infected chickens. Furthermore, the low level of haplotype and nucleotide diversity reported among *Et*AMA1 from Mpumalanga, South Africa, in this study showed that the *Et*AMA1 could be a good candidate for vaccine production. As such, screening for potential vaccine epitopes in this antigen that can induce lasting immune response is highly imperative for vaccine design against chicken coccidiosis.

3.5 References

Abdelli, N., Pérez, J.F., Vilarrasa, E., Luna, I.C., Melo-Duran, D., D'angelo, M., Solà-Oriol, D., 2020. Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge. Animals 10. https://doi.org/10.3390/ani10020259

Bachaya, H.A., Raza, M.A., Khan, M.N., Iqbal, Z., Abbas, R.Z., Murtaza, S., Badar, N., 2012. Predominance and detection of different eimeria species causing coccidiosis in layer chickens. J. Anim. Plant Sci. 22.

Blake, D.P., Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Jatau, I.D., Ayoade, S., Kawahara, F., Moftah, A., Reid, A.J., Adebambo, A.O., Zapata, R.Á., Rao, A.S.R.S., Thangaraj, K., Banerjee, P.S., Dhinakar-Raj, G., Raman, M., Tomley, F.M., 2015. Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. Proc. Natl. Acad. Sci. U. S. A. 112. https://doi.org/10.1073/pnas.1506468112

Blake, D.P., Worthing, K., Jenkins, M.C., 2020. Exploring Eimeria genomes to understand population biology: Recent progress and future opportunities. Genes (Basel). https://doi.org/10.3390/genes11091103

Bolton, 2015. The state of the South African poultry. [WWW Document]. URL https://scholar.google.co.uk/scholar?hl=en&as_sdt=0%2C5&q=Bolton%2C+T.+2015.+The+state+of+the+South+African+poultry+industry.+&btnG= (accessed 6.2.21).

Borgonovo, F., Ferrante, V., Grilli, G., Pascuzzo, R., Vantini, S., Guarino, M., 2020. A data-driven prediction method for an early warning of coccidiosis in intensive livestock systems: A preliminary study. Animals 10. https://doi.org/10.3390/ani10040747

Brunet, T.D.P., Ford Doolittle, W., Bielawski, J.P., 2021. The role of purifying selection in the origin and maintenance of complex function. https://doi.org/10.1016/j.shpsa.2021.03.005

Chenet, S.M., Branch, O.H., Escalante, A.A., Lucas, C.M., Bacon, D.J., 2008. Genetic diversity of vaccine candidate antigens in Plasmodium falciparum isolates from the Amazon basin of Peru 1, 1–11. https://doi.org/10.1186/1475-2875-7-93

DAFF, 2013. National aquaculture policy framework for South Africa. Natl. Aquac. policy Framew. South Africa 40.

Debbou-Iouknane, N., Benbarek, H., Ayad, A., 2018. Prevalence and aetiology of coccidiosis in broiler chickens in bejaia province, Algeria. Onderstepoort J. Vet. Res. 85. https://doi.org/10.4102/ojvr.v85i1.1590

Duszynski, D.W., Wilber, P.G., 1997. A Guideline for the Preparation of Species Descriptions in the Eimeriidae Author (s): Donald W . Duszynski and Patricia G . Wilber Published by : Allen Press on behalf of The American Society of Parasitologists Stable URL : https://www.jstor.org/stable/ 83, 333–336.

Eckford, S., Grace, K., Harris, C., Reeves, H., 2013. UK Veterinary Antibiotic Resistance and Sales Surveillance: UK-VARSS 2013. UK Vet. Antibiot. Resist. Sales Surveill. UK VARSS 132.

Fatoba, A.J., Zishiri, O.T., Blake, D.P., Peters, S.O., Lebepe, J., Mukaratirwa, S., Adeleke, M.A., 2020. Study on the prevalence and genetic diversity of eimeria species from

broilers and free-range chickens in kwazulu-natal province, south africa. Onderstepoort J. Vet. Res. 87. https://doi.org/10.4102/ojvr.v87i1.1837

Gharekhani, J., Sadeghi-Dehkordi, Z., Bahrami, M., 2014. Prevalence of Coccidiosis in Broiler Chicken Farms in Western Iran. J. Vet. Med. 2014. https://doi.org/10.1155/2014/980604

Han, H., Xue, P., Dong, H., Zhu, S., Zhao, Q., Huang, B., 2016. Screening and characterization of apical membrane antigen 1 interacting proteins in Eimeria tenella. Exp. Parasitol. 170. https://doi.org/10.1016/j.exppara.2016.09.014

Huang, X., Liu, J., Tian, D., Li, W., Zhou, Z., Huang, J., Song, X., Xu, L., Yan, R., Li, X., 2018. The molecular characterization and protective efficacy of microneme 3 of Eimeria mitis in chickens. Vet. Parasitol. 258. https://doi.org/10.1016/j.vetpar.2018.06.020

Jiang, L., Lin, J., Han, H., Dong, H., Zhao, Q., Zhu, S., Huang, B., 2012. Identification and characterization of eimeria tenella apical membrane antigen-1 (ama1). PLoS One 7. https://doi.org/10.1371/journal.pone.0041115

Kumar, S., Garg, R., Moftah, A., Clark, E.L., Macdonald, S.E., Chaudhry, A.S., Sparagano, O., Banerjee, P.S., Kundu, K., Tomley, F.M., Blake, D.P., 2014. An optimised protocol for molecular identification of Eimeria from chickens. Vet. Parasitol. 199. https://doi.org/10.1016/j.vetpar.2013.09.026

Kundu, K., Garg, R., Kumar, S., Mandal, M., Tomley, F.M., Blake, D.P., Banerjee, P.S., 2017. Humoral and cytokine response elicited during immunisation with recombinant Immune Mapped protein-1 (EtIMP-1) and oocysts of Eimeria tenella. Vet. Parasitol. 244. https://doi.org/10.1016/j.vetpar.2017.07.025

Kvičerová, J., Hypša, V., 2013. Host-Parasite Incongruences in Rodent Eimeria Suggest Significant Role of Adaptation Rather than Cophylogeny in Maintenance of Host Specificity. PLoS One 8. https://doi.org/10.1371/journal.pone.0063601

Lawal, J.R., Jajere, S.M., Ibrahim, U.I., Geidam, Y.A., Gulani, I.A., Musa, G., Ibekwe, B.U., 2016. Prevalence of coccidiosis among village and exotic breed of chickens in Maiduguri, Nigeria. Vet. World 9. https://doi.org/10.14202/vetworld.2016.653-659

Li, Y., He, L., Li, J., Chen, J., Liu, C., 2018. Genome-Wide Identification , Characterization , and Expression Profiling of the Legume BZR Transcription Factor Gene Family 9, 1–14. https://doi.org/10.3389/fpls.2018.01332

Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25. https://doi.org/10.1093/bioinformatics/btp187

Liu, L., Huang, X., Liu, J., Li, W., Ji, Y., Tian, D., Tian, L., Yang, X., Xu, L., Yan, R., Li, X., Song, X., 2017. Identification of common immunodominant antigens of Eimeria tenella, Eimeria acervulina and Eimeria maxima by immunoproteomic analysis. Oncotarget 8. https://doi.org/10.18632/oncotarget.16824

Lumkul, L., Sawaswong, V., Simpalipan, P., Kaewthamasorn, M., Harnyuttanakorn, P., Pattaradilokrat, S., 2018. Unraveling haplotype diversity of the apical membrane antigen-1 gene in Plasmodium falciparum populations in Thailand. Korean J. Parasitol. 56, 153–165. https://doi.org/10.3347/kjp.2018.56.2.153

Ma, D., Huang, Y., Ma, C., Zhang, L., Wang, J., Wang, D., Li, J., Dalloul, R.A., 2019. Eimeria tenella: Specific EtAMA1-binding peptides inhibit sporozoite entry into host

cells. Poult. Sci. 98. https://doi.org/10.3382/ps/pez298

Mehrizi, A.A., Sepehri, M., Karimi, F., Djadid, N.D., Zakeri, S., 2013. Population genetics, sequence diversity and selection in the gene encoding the Plasmodium falciparum apical membrane antigen 1 in clinical isolates from the south-east of Iran. Infect. Genet. Evol. 17, 51–61. https://doi.org/10.1016/j.meegid.2013.03.042

Nei, M., Gojoborit, T., 1986. Simple Methods for Estimating the Numbers of Synonymous and Nonsynonymous Nucleotide Substitutions ' 3, 418–426.

Pastor-Fernández, I., Kim, S., Billington, K., Bumstead, J., Marugán-Hernández, V., Küster, T., Ferguson, D.J.P., Vervelde, L., Blake, D.P., Tomley, F.M., 2018. Development of cross-protective Eimeria-vectored vaccines based on apical membrane antigens. Int. J. Parasitol. 48. https://doi.org/10.1016/j.ijpara.2018.01.003

Peek, H.W., Landman, W.J.M., 2011. Coccidiosis in poultry: Anticoccidial products, vaccines and other prevention strategies. Vet. Q. 31. https://doi.org/10.1080/01652176.2011.605247

Rafiqi, S.I., Garg, R., Ram, H., Reena, K.K., Asari, M., Kumari, P., Kundave, V.R., Singh, M., Banerjee, P.S., 2019. Immunoprophylactic evaluation of recombinant gametocyte 22 antigen of Eimeria tenella in broiler chickens. Parasitol. Res. 118. https://doi.org/10.1007/s00436-018-06198-2

Reid, A.J., Blake, D.P., Ansari, H.R., Billington, K., Browne, H.P., Bryant, J., Dunn, M., Hung, S.S., Kawahara, F., Miranda-Saavedra, D., Malas, T.B., Mourier, T., Naghra, H., Nair, M., Otto, T.D., Rawlings, N.D., Rivailler, P., Sanchez-Flores, A., Sanders, M., Subramaniam, C., Tay, Y.L., Woo, Y., Wu, X., Barrell, B., Dear, P.H., Doerig, C., Gruber, A., Ivens, A.C., Parkinson, J., Rajandream, M.A., Shirley, M.W., Wan, K.L., Berriman, M., Tomley, F.M., Pain, A., 2014. Genomic analysis of the causative agents of coccidiosis in domestic chickens. Genome Res. 24. https://doi.org/10.1101/gr.168955.113

SAPA, 2019. SOUTH AFRICAN POULTRY ASSOCIATION 2019 INDUSTRY PROFILE.

Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009. Genomic analysis of eimeria spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. J. Parasitol. 95. https://doi.org/10.1645/GE-1898.1

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry Coccidiosis: Design and Interpretation of Vaccine Studies. Front. Vet. Sci. https://doi.org/10.3389/fvets.2020.00101

Stats-SA, 2020. Mid-year population estimates.

Suprihati, E., Yunus, M., 2018. Evaluation of the antigenicity and immunogenicity of eimeria tenella by reproductive index and histopathological changes of cecal coccidiosis virulent live vaccine in broiler chickens. African J. Infect. Dis. 12. https://doi.org/10.2101/Ajid.12v1S.15

Tamura K, Peterson D, Stecher G, Filipski A, & Kumar S (2013) MEGA6: Molecular Evolutionary Genetic Analysis version 6.0 Molecular Biology and Evolution 30: 2725–2729.

Tian, L., Li, W., Huang, X., Tian, D., Liu, J., Yang, X., Liu, L., Yan, R., Xu, L., Li, X., Song, X., 2017. Protective efficacy of coccidial common antigen glyceraldehyde 3-phosphate dehydrogenase (GAPDH) against challenge with three Eimeria species. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01245

Tomley, F., 1997. Techniques for isolation and characterization of apical organelles from Eimeria tenella sporozoites. Methods A Companion to Methods Enzymol. 13. https://doi.org/10.1006/meth.1997.0509

Tyler, J.S., Treeck, M., Boothroyd, J.C., 2011. Focus on the ringleader: The role of AMA1 in apicomplexan invasion and replication. Trends Parasitol. https://doi.org/10.1016/j.pt.2011.04.002

Wang, Y.N., Lin, M., Liang, X.Y., Chen, J.T., Xie, D. De, Wang, Y.L., Ehapo, C.S., Eyi, U.M., Huang, H.Y., Wu, J.L., Xu, D.Y., Chen, Z.M., Cao, Y.L., Chen, H. Bin, 2019. Natural selection and genetic diversity of domain i of Plasmodium falciparum apical membrane antigen-1 on Bioko Island. Malar. J. 18, 1–16. https://doi.org/10.1186/s12936-019-2948-y

Witcombe, D.M., Smith, N.C., 2014. Strategies for anti-coccidial prophylaxis, in: Parasitology. Cambridge University Press, pp. 1379–1389. https://doi.org/10.1017/S0031182014000195

Yan, M., Cui, X., Zhao, Q., Zhu, S., Huang, B., Wang, L., Zhao, H., Liu, G., Li, Z., Han, H., Dong, H., 2018. Molecular characterization and protective efficacy of the microneme 2 protein from Eimeria tenella. Parasite 25. https://doi.org/10.1051/parasite/2018061

CHAPTER 4

In silico design of multiepitope-based vaccine of *Eimeria tenella* Apical Membrane Antigen 1 against Chicken Coccidiosis.

Abstract

The sporozoite-specific *Eimeria tenella* apical membrane antigen 1 (*Et*AMA1) is a vital vaccine candidate that can induce immune responses against coccidiosis infection. Attenuated vaccines have been the gold standard to fight the spread of coccidiosis infection; however, this type of vaccine is expensive and time-consuming. Therefore, there is a need to introduce immunoinformatics to design a vaccine competent in inducing innate and adaptive immune responses inside a host's body. The current study was designed to detect conserved B-cell and T-cell epitopes of *Et*AMA1, which could play a significant role in inducing the immune reactions against coccidiosis chickens. Hundred and twenty-eight *Et*AMA1 protein sequences were subjected to several tests, and out of this 6 B-cell and 9 T-cell epitopes were predicted and found to be immunogenic and non-allergen. The multiepitope vaccine (MEV) construct consists of 6 linear B-cell, 3 $CD8^+$ T-cell and 6 $CD4^+$ T-cell epitopes joined together by linkers and adjuvants. The designed MEV was 311 amino acids long. Both adjuvant and linkers were used to increase the immunogenicity of the MEV. The designed MEV was highly antigenic, non-allergen and showed a strong binding affinity with TLR4. This study shows that the vaccine construct, once validated experimentally, could serve as an effective vaccine against chicken coccidiosis.

Keywords: Coccidiosis, Epitopes, *Et*AMA1, Immunoinformatics, Vaccine

4.0 Introduction

Coccidiosis is a parasitic infection caused by *Eimeria* (Jordan et al., 2018). Due to the lack of appropriate anticoccidial drugs, expensive and time-consuming live-attenuated vaccines (Lawal et al., 2016; Soutter et al., 2020), immunoinformatics can be used instead to design a peptide-based vaccine against this deadly parasitic disease. This approach has been known to play a vital role in the identification of new antigenic epitopes that can be used when designing a vaccine for many infectious diseases caused by viruses, bacteria, fungi and parasites (Raoufi et al., 2020). Peptide-based vaccines are considered as cost-effective and require less time for development compared to traditional methods (Haraz et al., 2017; Ali et al., 2017; Awad Elkareem et al., 2017). The vaccine against *Neisseria meningitis* was the first vaccine to be designed following the immunoinformatics approach, and it was developed successfully afterwards (Adu-Bobie et al., 2003). The same methodology has been used in subsequent years to design peptide vaccines based on linear B and T cell epitopes (Florian et al., 2014). The design of a multiepitope vaccine through immunoinformatics approaches is known to produce adequate results (Reche et al., 2014). The feasibility, safety and accuracy of these were discussed via various studies (Bande et al., 2016; Wang et al., 2017).

Pandey *et al*., 2018 stated that when designing a viable multiepitope vaccine, (1) different antigenic determinants must be selected, (2) adjuvants must be added to increase host immune response in the host (Pandey et al., 2018a). These novel techniques have been successfully used to design B-cell and T-cell epitope-based vaccine against different avian diseases such as Infectious bronchitis (Al-Khafaji and Mahmood, 2018), Newcastle disease Virus (NDV) (Putri et al., 2019) and Avian Encephalomyelitis (AE) (Almofti et al., 2021). However, researchers have stated that there are challenges when designing vaccine against chicken diseases, such as the lack of chicken MHC alleles. As a result, Human MHC 1 alleles are used instead (Tan et al., 2016). Still, to date, no study has been reported on the development of a subunit vaccine against chicken coccidiosis using immunoinformatics. Hence, the current study aims to use Mpumalanga *Et*AMA1 plus NCBI *Et*AMA1 sequences to develop a potential cost-effective multiepitope vaccine against coccidiosis through immunoinformatics approaches.

4.1 Materials and Methods

4.1.1 Retrieval of targeted protein sequences, antigenicity prediction and transmembrane helix

A total of 128 protein sequences of *Et*AMA1 were used in the current study. Thirty-five (35) were from Mpumalanga province, and 93 were retrieved in a FASTA format from the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The sequences were aligned with conserved $CD8^+$ (PDB: 1AKJ_D) and $CD4^+$ (PDB: 3S4S_G) using ClustalW server (https://www.genome.jp/tools-bin/clustalw) (Thompson et al., 2003). Protein sequences aligned with $CD8^+$ and $CD4^+$ sequences (with the minimum number of 15 amino acids) were selected for further analysis. The antigenicity of all the sequences was tested by submitting the sequences at the VaxiJen v20 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Doytchinova and Flower, 2007) with a threshold of 0.4. Then the antigenic sequences were further subjected to the TMHMM v2.0 server (http://www.cbs.dtu.dk/services/TMHMM/) to predict transmembrane domains of *Et*AMA1.

4.1.2 Prediction of $CD8^+$ epitopes and their major histocompatibility complex (MHC) class I binding allele

Potential epitopes in FASTA format that have passed the antigenicity and membrane tests were further submitted at NetCTL v1.2 software (http://www.cbs.dtu.dk/services/NetCTL/) (Larsen et al., 2007) to predict the nonamers sequences which are capable of inducing $CD8^+$ epitopes. The nonamers sequences above 0.5 thresholds were subjected to the Immune Epitope Database (IEDB) MHC-I binding tool (http://tools.iedb.org/mhci/) (Andreatta and Nielsen, 2015) to bind MHC-1 (HLA) alleles based on the stabilized matrix method (SMM). Due to the lack of chicken MHC in most immunoinformatics software, human MHC (HLA) was selected. The rationale for the selection of human MHC as a substitute was based on the previous report that showed that anchor residues in chicken BF haplotypes are similar to residues anchored on mammalian MHC-I, especially those with 8-9mer in size (Tan et al., 2016). The peptide sequences that have IC50 value < 250 were selected for further analysis. Prospective vaccine components should be antigenic and immunogenic, i.e., they can trigger an immune response. Therefore, the antigenicity and immunogenicity were evaluated by submitting the sequences at the VaxiJen v2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Doytchinova and Flower, 2007) with a 0.5 threshold and IEDB MHC-class I immunogenicity

tool (http://tools.iedb.org/immunogenicity/) (Calis et al., 2013) respectively. Non-immunogenic epitope sequences denoted by negative values were excluded.

4.1.3 Prediction of CD4+ epitopes and their major histocompatibility complex (MHC) class II binding allele

The membrane sequences in FASTA format was used to generate $CD4^+$ epitopes. The 15-mer $CD4^+$ epitopes and their corresponding MHC-II alleles were predicted through the IEDB MHC-II binding tool (http://tools.iedb.org/mhcii/) using the SMM-align prediction method. The epitopes with the IC50 value <250 were selected and used for checking protective antigenicity scores using VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a 0.5 threshold. The epitopes with antigenicity scores above 0.5 were selected and used for further analysis as done elsewhere (Rodr et al., 2020).

4.1.4 Conservancy and allergenicity prediction of the CD8 and CD4 T epitope sequences

The immunogenic $CD8^+$ and antigenic $CD4^+$ epitope sequences were subjected to the IEDB Analysis Resource (http://tools.iedb.org/conservancy/) (Bui et al., 2007) for the prediction of epitope Conservancy. The $CD4^+$ epitopes, which overlapped with $CD8^+$ T-cell epitopes with 100% conservancy, were selected and tested for allergenicity using AllerTOPV.2.0 (https://www.ddg-pharmfac.net/AllerTOP/).

4.1.5 Analysis of IFN-γ and IL-4 inducer property of the $CD4^+$ T-cell epitopes and conservancy

Conserved $CD4^+$ epitopes were further used to test if they are inducer or non-inducer of IFN-γ and IL-4, respectively. The IFNepitope tool (http://crdd.osdd.net/raghava/ifnepitope/predict.php) (Dhanda et al., 2013) was used to predict if the conserved $CD4^+$ epitope has the capacity to induce IFN-γ. The SVM based method was used. While the IL-4 inducer was predicted through the IL4pred tool (https://webs.iiitd.edu.in/raghava/il4pred/design.php) (Dhanda et al., 2013) using the SVM prediction method with 0.1 thresholds. The $CD4^+$ epitopes that were able to induce both IFN-γ and IL-4 were selected for further analysis. Conservancy analysis was done between the conserved $CD8^+$ epitope and $CD4^+$ epitope inducing both IFN-γ and IL-4 using IEDB analysis resource (http://tools.iedb.org/conservancy/) (Bui et al., 2007). The $CD8^+$ and $CD4^+$ conserved non-allergen epitopes were used for docking.

4.1.6 Linear B-cell epitope prediction

The FASTA sequences that have passed antigenicity and membrane tests were submitted to the online server ABCpred (http://crdd.osdd.net/raghava/abcpred/) with a threshold of 0.9. The generated B-cell epitopes were then tested for antigenicity with a threshold of 1.0 and allergenicity. B-cell epitopes with an antigenic score greater than 1 and non-allergen were further used for molecular docking.

4.1.7 Construction of a Multiepitope vaccine (MEV)

The multiepitope vaccine was constructed using adjuvant, linkers, B-cell, $CD4^+$ and $CD8^+$ T-cell epitopes. Beta-defensin (XP_030668226), an immunological adjuvant (consisting of 45 amino acid long), was attached to the vaccine construct's N-terminal site through an EAAAK linker to potentiate antigen-specific immune responses. Other linkers that were used to join adjacent epitopes includes AAY, GPGPG and KK linker. Beta-defensin and B-cell epitopes were linked by the EAAAK linker as previously described (Arai et al., 2001). In contrast, KK was used to join B-cell epitopes with $CD8^+$ epitopes. AAY and GPGPG linkers were used to join $CD8^+$ and $CD4^+$ T-cell epitopes, respectively. The final sequence of the designed MEV candidate consisted of the adjuvant, linkers, B-cells, $CD8^+$ and $CD4^+$ T-cell epitopes that were tested for allergenicity and antigenicity. The constructed vaccine was then submitted at trRosetta (https://yanglab.nankai.edu.cn/trRosetta/) to generate its 3D structure. The predicted 3D structure of the MEV was later submitted at the Galaxy refine server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) (Heo et al., 2013) for refinement.

4.1.8 Molecular Docking

Chicken MHC-alleles- BF2*2101 (PDB: 4D0C) and pBL2*019:01 (PDB: 6KVM) were used for molecular docking of 3 $CD8^+$ and 6 $CD4^+$ T-cell epitopes. The alleles were subjected to RCSB Protein Data Bank (http://www.rcsb.org/) to download the PDB structures, then viewed in UCSF Chimera 1.14. Likewise, a docking of constructed multiepitope vaccine with Toll-like receptor 4 (TLR4, PDB ID: 4G8A) was also performed. The Molecular docking was done using ATTRACT site (http://www.attract.ph.tum.de/services/ATTRACT/peptide.html) (Vries et al., 2017) and completed on Centre for High-Performance Computing (CHPC). The ATTRACT site generated 50 structures for each epitope. Epitopes with the lowest binding energy level were selected as the best structure and visualized in VMD 1.9.3 (Humphrey et al., 1996) software then combined in one structure using Chimera 1.14 (Pettersen et al., 2004).

4.1.9 Validation of refined 3D structure

The refined 3D structure of the constructed MEV was further submitted at ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) to detect any possible errors on the refined vaccine 3D structure. The web server provides information on the total quality score of the inputted three-dimensional structure (Wiederstein and Sippl, 2007). The tertiary structure of the protein was then validated using the Ramachandran plot analysis (https://swissmodel.expasy.org/assess).

4.2 Results

4.2.1 Selection of sequences with a capacity to induce an immune response

The sequences aligned with $CD8^+$ and $CD4^+$ T-cells were tested for antigenicity and membrane tests. Only 39 sequences passed the tests and were used for further analysis.

4.2.2 $CD8^+$ T-cell epitopes prediction and evaluation of their Immunogenicity

In all, 3 tops immunogenic (positive immunogenicity score) and antigenic $CD8^+$ epitopes were selected as part of the vaccine design (Table 1).

Table 1: Predicted $CD8^+$ T-cell epitopes of *Et*AMA1, which overlapped 100% with $CD4^+$ T-cell epitopes and interacting with different MHC-I HLA alleles

Epitopes	HLA alleles	IC_{50} (nM)	Antigenicity score	Allergenicity
CPAAVVAPA	HLA-B*35:01	93.8814	0.6063	Non-allergen
	HLA-B*07:02	249.9712		
LENLVVGPV	HLA-B*40:01	157.0001	0.6907	Non-allergen
VSLRPHLVY	HLA-B*58:01	191.1172	0.6570	Non-allergen

4.2.3 Prediction and selection of CD4+ T-cell epitopes

The MHC-II specific $CD4^+$ T-cell epitopes were obtained using the IEDB web server. Two hundred ninety-one (291) unique 15-mer $CD4^+$ epitopes were sorted based on their IC50 value, which was then tested for antigenicity. Of the 291 epitopes tested for antigenicity, only 120 passed the test and were further subjected to the allergenicity test. The epitopes that were antigenic and non-allergen were subjected to IFN- and IL-4 prediction, and only 6 $CD4^+$ T-cell epitopes can induce both IFN-γ and IL-4 (Table 2).

Table 2: Predicted potential $CD4^+$ T-cell epitopes of *Et*AMA1 interacting with different MHC-II HLA alleles

Epitopes	HLA alleles	IC_{50} (nM)	Antigenicity score	Allergenicity	IFN-inducer	IL4 inducer
LKSVSLRPHLVYGSA	HLA-DRB1*01:01	41.00	0.6527	Non-allergen	Yes	Yes
LVVGPVCPAAVVAPA	HLA-DRB1*09:01	191.00	0.9829	Non-allergen	Yes	Yes
PLKSVSLRPHLVYGS	HLA-DRB1*01:01	43.00	0.7992	Non-allergen	Yes	Yes
VVGPVCPAAVVAPAV	HLA-DRB1*01:01	224.00	0.8749	Non-allergen	Yes	Yes
KSVSLRPHLVYGSAY	HLA-DRB1*01:01	102.00	0.6540	Non-allergen	Yes	Yes
LENLVVGPVCPAAVV	HLA-DRB1*09:01	184.00	0.7038	Non-allergen	Yes	Yes
	HLA-DRB1*01:01	222.00				

4.2.4 Molecular docking of $CD8^+$ and $CD4^+$ T-cell epitopes with their respective Alleles

The molecular docking of $CD8^+$ and $CD4^+$ T-cell epitopes with their respective alleles are presented in Figure 1. The docking of $CD8^+$ with MHC-BF2*2101 (Fig. 1A) and (B) shows the docking of $CD4^+$ with MHC- pBL2*019:01. Both Fig. 1A and 1B showed that the $CD8^+$ and $CD4^+$ T-cells were successfully bound to their respective alleles.

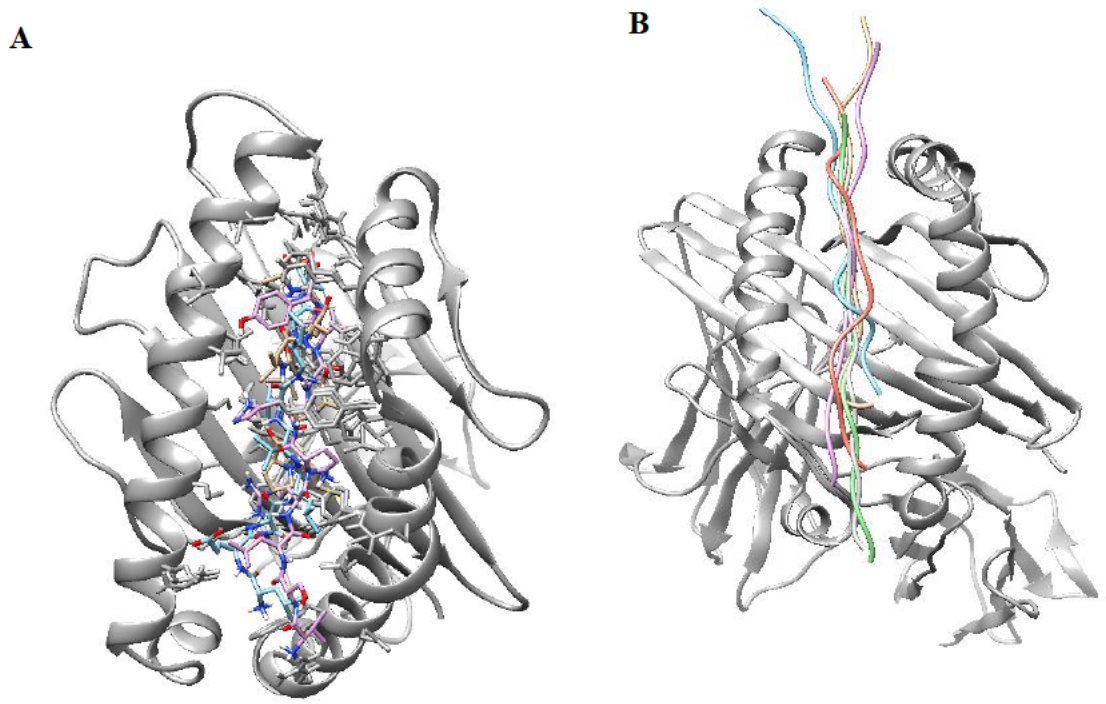

Fig 1: Image showing the binding of $CD8^+$ and $CD4^+$ T-cell epitopes with their respective chicken MHC-alleles

4.2.5 Linear B-cell epitope prediction

A total of 16 B-cell epitopes were obtained via the ABCpred server. These epitopes were subjected to antigenicity and allergenicity. Only 6 (Table 3) B-cell epitopes found to be antigenic and non-allergen. These epitopes were considered for vaccine construction.

Table 3: Linear B-cell epitopes of EtAMA1 with their antigenicity and allergenicity

B-cell epitopes	ABCprep score	VaxiJen score	Conservancy	Allergenicity
AASSDASTDSNPFMQP	0.90	1.2110	100%	Non-allergen
PGGGTGTTGGGGGCTG	0.93	2.8037	100%	Non-allergen
PSDAITPGPCSSNPCK	0.91	1.2873	100%	Non-allergen
SAMGSASTSGNPFQAN	0.90	1.2045	100%	Non-allergen
SSSIVSPRSPVPSSNS	0.95	1.0206	100%	Non-allergen
TGGGTGTTGGGGGCTG	0.92	3.1377	100%	Non-allergen

4.2.6 Multiepitope vaccine design

The multiepitope vaccine was 311 amino acids long and joined by using linkers. Multiepitope vaccine was designed by joining B-cells, $CD8^+$ and $CD4^+$ T-cell epitopes as reported elsewhere (Pandey et al., 2018b). Beta-defensin was used as an adjuvant to increase the immunogenicity of the vaccine (Mohan et al., 2013). Each epitope was separated by a linker (EAAAK, AAY, GPGPG and KK). In total, 15 linkers added to design the multiepitope subunit vaccine: 1 EAAAK linker, 6 KK linkers, 3 AAY linkers and 5 GPGPG linkers. The sequence of the MEV is shown in Fig. 2A, while the refined 3D structure is presented in Fig. 2B. A subunit vaccine should be non-allergen and immunogenic (Narula et al., 2018). The multiepitope vaccine designed was found to be non-allergen and antigenic with a probabilistic score of 0.9674.

A

GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKKEAAAKAASSDAS
TDSNPFMQPKKPGGGTGTTGGGGGCTGKKPSDAITPGPCSSNPCKKKSAMGSASTSG
NPFQANKKSSSIVSPRSPVPSSNSKKTGGGTGTTGGGGGCTGKKCPAAVVAPAAAYL
ENLVVGPVAAYVSLRPHLVYAAYLENLVVGPVCPAAVVGPGPGLKSVSLRPHLVYG
SAGPGPGLVVGPVCPAAVVAPAGPGPGKSVSLRPHLVYGSAYGPGPG
PLKSVSLRPHLVYGSGPGPGVVGPVCPAAVVAPAV

B

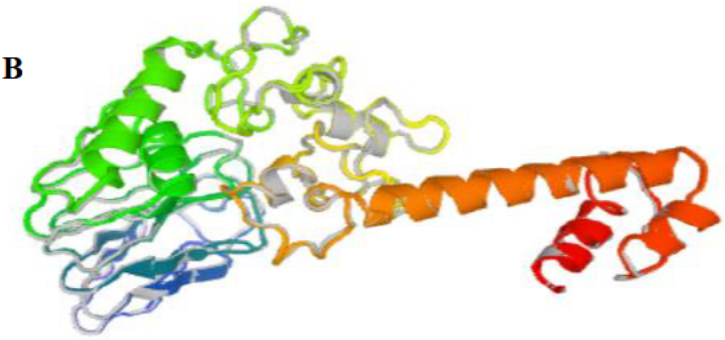

Fig. 2: The sequence and refined 3D structure of the designed MEV. (A) MEV construct sequence (B) Refined 3D structure of MEV construct.

4.2.7 Validation of a designed vaccine 3D structure and Molecular docking of multiepitope vaccine with Toll-like receptor (TLR4)

Ramachandran plot analysis (Fig. 3A) revealed that 94.46% of residues were mostly found in favoured regions, 1.23% in allowed and 0.00% unallowed regions. ProsA-web showed a Z-score of -5.21 for the designed vaccines' inputted refined tertiary structure (Fig. 3B). Molecular docking of the constructed subunit vaccine with TLR-4 receptor resulted in 50 models. All the models had different energy score. However, model 36 was selected as the best dock-model due to its lowest energy score of -279.024. The multiepitope is highlighted in purples, while the TLR4 is highlighted in grey (Fig. 4)

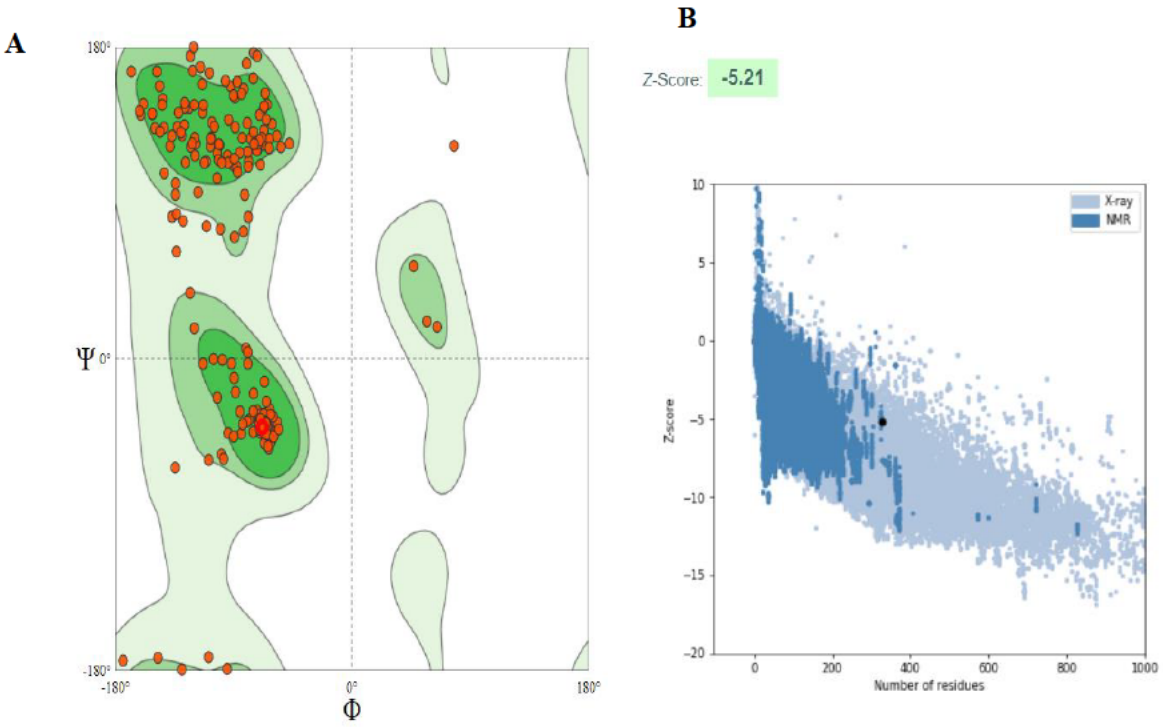

Fig. 3: The Ramachandran plot (A) and Validation of the refined 3D structure (B) showing a *Z*-score of -5.21.

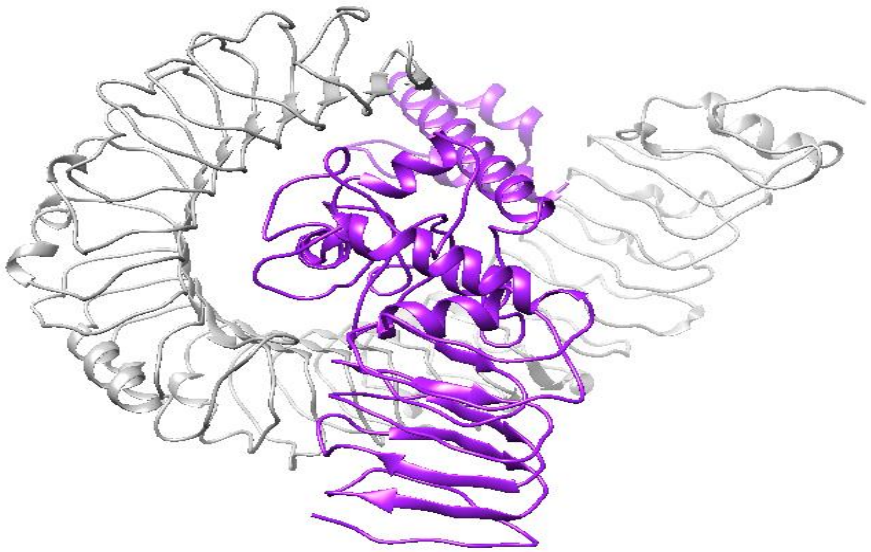

Fig. 4. Molecular docking of a constructed multiepitope vaccine (purple) with TLR-4 (grey) receptor.

4.3 Discussion

Eimeria infections cause a severe public health problem and are of great economic importance globally. Many strategies have been implemented to control coccidiosis. However, due to the increasing drug resistance of *Eimeria* and the high production cost of the live vaccine (Clark et al., 2017), the development of cost-effective vaccines that can induce protection against several species of *Eimeria* are urgently required to combat this disease. Bioinformatic techniques have opened a new era on new strategies in advanced vaccine design against different diseases (Kaliamurthi et al., 2018; Zhao and Wei, 2018). The recent exploration of immunoinformatics strategies plays a pivotal role in the robust screening of many genomes to predict specific epitopes that can stimulate the T-cell immune responses economically (Gao and Jakobsen, 2000; Kamthania and Sharma, 2015; Zhao and Wei, 2018). The current study used *Et*AMA1 protein sequences to design a multiepitope subunit vaccine for chicken coccidiosis. An ideal multiepitope based subunit vaccine should include both B- and T-cell epitopes for encouraging a complete system of immune responses (Tahir et al., 2020).

A total of 9 top-most immunogenic and antigenic T-cell epitopes and 6 B-cell epitopes were used for vaccine design. The $CD4^+$ and $CD8^+$ epitopes were 100% overlap and conserved to each other. This showed that the selected the $CD4^+$ and $CD8^+$ epitopes were good candidates for designing a potent vaccine candidate. A previous and recent study has also shown high overlaps of peptide binding to various HLAs (Lim et al., 2021; Zhao et al., 2013). The T-cell immune response is considered an essential response compared to B-cell immune response (Black et al., 2010). The stimulation of the helper T-cells is germane for potent vaccine development. The epitopes of $CD4^+$ helper T-cells can induce or provoke the helper T-cells to produce cytokines. The helper T-cells can produce an array of cytokines such as interleukin-4 (IL-4), interferon-gamma (IFN-γ), etc., to challenge and fight intracellular pathogens such as parasites when provoked by the epitopes of the $CD4^+$ helper T-cells (Adhikari and Rahman, 2017a). The predicted $CD4^+$ T-cells epitopes were subjected to rigorous tests to determine whether they can induce IFN-γ and IL-4 or not. Fortunately, all the predicted $CD4^+$ epitopes were found to be IFN-γ and IL-4 inducers, which indicate that the predicted CD^+ T-cells epitopes are potential vaccine candidate in accordance with the previous reports (Black et al., 2010; Adhikari and Rahman, 2017b). Additionally, the $CD4^+$ T and $CD8^+$ T-cell responses play the most crucial part in anticoccidial immunity (Lillehoj, 1998; Kim et al., 2019). Though the specific role of the T-cell subpopulation in coccidiosis still need to be clarified, T-cells provide the most significant protection against avian coccidiosis (Kim et al., 2019).

Consequently, it is imperative to design a vaccine that has the ability to induce both $CD4^+$ T and $CD8^+$ T-cell responses.

The B-cells are essential components of the adaptive immune system capable of producing antibodies against attacking pathogens. Antibodies are becoming a common and valuable therapeutic agent against contagious diseases (Sormanni et al., 2015; Chowdhury et al., 2018). T cells are more responsible for acquired immunity against coccidiosis than B cells. Both T and B lymphocyte subtypes that mediate antigen specific immune responses are involved adaptive immunity, which is crucial in imparting protection against secondary infections (Tellez et al., 2014). However, the specific role of B-cells against coccidiosis is debated (Wallach, 2010). The constructed vaccine consists of B-cell epitopes; this shows that it has higher chances of controlling coccidiosis. Molecular docking was done to explore the binding affinity between alleles and the predicted $CD8^+$ and $CD4^+$ epitopes. Docking analysis showed that the predicted epitopes successfully bind with their corresponding alleles, indicating that they can elicit immune responses.

In this study, Beta-defensin was used as an adjuvant and attached at the N-terminal site of the vaccine through EAAAK linker followed by B-cell and T-cell epitopes. The adjuvant, together with the epitopes was joined together with linkers. Beta-defensins play pivotal roles in both natural and acquired immune responses (Tani et al., 2000; Zhang et al., 2010). Beta-defensin is a multifunctional peptide that acts as an immunomodulator (Hoover et al., 2003) manage the cross-talk between host and pathogens, preventing pathogen-associated inflammation and disease (Meade and O'Farrelly, 2019) and reduced and/or weakens proinflammatory cytokine responses (Kohlgraf et al., 2010; Meade and O'Farrelly, 2019). Beta-defensin can enhance the immunogenicity of a vaccine (Narula et al., 2018); this warrants its usage in the current study.

The constructed multiepitope vaccine was found to be immunogenic and non-allergen. This shows that the vaccine construct can induce immune responses and is safe to use i.e. it cannot cause any allergic response. The Ramachandran plot analysis showed that more residues (94.46%) were found in favoured regions and 1.23% in allowed and 0.00% unallowed regions, while the Z-score was -5.21. This shows that the overall quality of the structure is good and in accordance with other reports (Khatoon et al., 2017; Pandey et al., 2018a; Nain et al., 2019; Almofti et al., 2021). Docking analysis shows a stable interaction between the vaccine construct and TLR4. The TLR4 is one of the most important innate immune receptors, which plays a crucial part against coccidiosis (Zhou et al., 2013).

Several studies have been reported on multiepitope vaccines design using immunoinformatics approaches (Ikram et al., 2018; Pandey et al., 2019). The proposed multiepitope vaccine in the current study consists of B-cell and T-cell epitopes along with an adjuvant, which has the capacity to stimulate innate and adaptive immune responses and a potential candidate for coccidiosis vaccine development.

4.4 Conclusion

Coccidiosis has emerged as a severe health issue that affects livestock, especially chickens worldwide. Lack of cost-effective vaccines and increasing resistance to anticoccidial drugs necessitates the need to develop an alternative vaccine candidate to control this deadly disease. In this study, we have successfully designed a multiepitope vaccine using an immunoinformatics approach. The overlapping $CD8^+$ and $CD4^+$ T-cell epitopes from *Et*AMA1 were predicted and successfully identified via immunoinformatics strategies. The identified $CD8^+$ and $CD4^+$ T-cell epitopes are antigenic and immunogenic and have cytokine (IFN-γ and IL-4) inducing abilities. The CD8+ and CD4+ T-cell epitopes used in the MEV construct also have a high binding capacity to their HLA-alleles. The predicted B-cell used in the MEV design is also antigenic. We presume that the predicted multiepitope, when validated experimentally, could serve as an effective vaccine against chicken coccidiosis across the globe.

4.5 References

Abu haraz, A.H., Abd elrahman, K.A., Ibrahim, M.S., Hussien, W.H., Mohammed, M.S., Badawi, M.M., Ahmed Salih, M., 2017. Multi Epitope Peptide Vaccine Prediction against Sudan Ebola Virus Using Immuno-Informatics Approaches. Adv. Tech. Biol. Med. 05. https://doi.org/10.4172/2379-1764.1000203

Adhikari, U.K., Rahman, M.M., 2017a. Infection , Genetics and Evolution Overlapping CD8 + and CD4 + T-cell epitopes identi fi cation for the progression of epitope-based peptide vaccine from nucleocapsid and glycoprotein of emerging Rift Valley fever virus using immunoinformatics approach 56, 75–91. https://doi.org/10.1016/j.meegid.2017.10.022

Adhikari, U.K., Rahman, M.M., 2017b. Overlapping CD8 + and CD4 + T-cell epitopes identification for the progression of epitope-based peptide vaccine from nucleocapsid and glycoprotein of emerging Rift Valley fever virus using immunoinformatics approach. Infect. Genet. Evol. 56. https://doi.org/10.1016/j.meegid.2017.10.022

Adu-Bobie, J., Capecchi, B., Serruto, D., Rappuoli, R., Pizza, M., 2003. Two years into reverse vaccinology, in: Vaccine. https://doi.org/10.1016/S0264-410X(02)00566-2

Al-Khafaji, Z.M., Mahmood, A.B., 2018. Designing of Mlti-Epitope Vaccine Based on Vaccine Strains Against Iraqi and Variant 2 Infectious Bronchitis Viral Strains 4. https://doi.org/10.26479/2018.0403.33

Ali, M., Pandey, R.K., Khatoon, N., Narula, A., Mishra, A., 2017. Exploring dengue genome to construct a multi-epitope based subunit vaccine by utilizing immunoinformatics approach to battle against dengue infection. Sci. Rep. 1–13. https://doi.org/10.1038/s41598-017-09199-w

Almofti, Y., Ali, K., Mohammed, A., Eltilib, E.E.M., 2021. Structural Analysis of Avian Encephalomyelitis Virus Polyprotein for Structural Analysis of Avian Encephalomyelitis Virus Polyprotein for Development of Multi Epitopes Vaccine Using Immunoinformatics Approach. https://doi.org/10.22207/JPAM.15.1.20

Andreatta, M., Nielsen, M., 2015. Gapped sequence alignment using artificial neural networks : application to the MHC class I system.

Arai, R., Ueda, H., Kitayama, A., Kamiya, N., Nagamune, T., 2001. Design of the linkers which effectively separate domains of a bifunctional fusion protein 14, 529–532.

Awad Elkareem, M.A.E., Ahmed Osman, S., Mohamed, H.A., Elrahman Hassan, H.A., Abuharaz, A.H., Abd elrahman, K.A., Ahmed Salih, M., 2017. Prediction and Conservancy Analysis of Multiepitope Based Peptide Vaccine Against Merkel Cell Polyomavirus: An Immunoinformatics Approach. Immunome Res. 13. https://doi.org/10.4172/1745-7580.1000134

Bande, F., Arshad, S.S., Bejo, M.H., Kadkhodaei, S., Rahman Omar, A., 2016. Prediction and In Silico Identification of Novel B-Cells and T-Cells Epitopes in the S1-Spike Glycoprotein of M41 and CR88 (793/B) Infectious Bronchitis Virus Serotypes for Application in Peptide Vaccines. https://doi.org/10.1155/2016/5484972

Black, M., Trent, A., Tirrell, M., Olive, C., 2010. Advances in the design and delivery of peptide subunit vaccines with a focus on Toll-like receptor agonists. Expert Rev. Vaccines. https://doi.org/10.1586/erv.09.160

Bui, H., Sidney, J., Li, W., Fusseder, N., Sette, A., 2007. Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines 6, 1–6. https://doi.org/10.1186/1471-2105-8-361

Calis, J.J.A., Maybeno, M., Greenbaum, J.A., Weiskopf, D., Silva, A.D. De, 2013. Properties of MHC Class I Presented Peptides That Enhance Immunogenicity 9. https://doi.org/10.1371/journal.pcbi.1003266

Chowdhury, R., Allan, M.F., Maranas, C.D., 2018. OptMAVEn-2.0: De novo Design of Variable Antibody Regions Against Targeted Antigen Epitopes. Antibodies 7. https://doi.org/10.3390/antib7030023

Clark, E.L., Tomley, F.M., Blake, D.P., 2017. Are Eimeria Genetically Diverse, and Does It Matter? Trends Parasitol. https://doi.org/10.1016/j.pt.2016.08.007

Dhanda, S.K., Gupta, S., Vir, P., Raghava, G.P.S., 2013. Prediction of IL4 Inducing Peptides 2013.

Doytchinova, I.A., Flower, D.R., 2007. VaxiJen : a server for prediction of protective antigens , tumour antigens and subunit vaccines 7, 1–7. https://doi.org/10.1186/1471-2105-8-4

Florian, D.D., Shawan, M.M.A.K., Al Mahmud, H., Hasan, M.M., Parvin, A., Rahman, M.N., Badier Rahman, S.M., 2014. In Silico Modeling and Immunoinformatics Probing Disclose the Epitope Based PeptideVaccine Against Zika Virus Envelope Glycoprotein. Indian J. Pharm. Biol. Res. 2. https://doi.org/10.30750/ijpbr.2.4.10

Gao, G.F., Jakobsen, B.K., 2000. Molecular interactions of coreceptor CD8 and MHC class I: The molecular basis for functional coordination with the T-cell receptor. Immunol. Today. https://doi.org/10.1016/S0167-5699(00)01750-3

Heo, L., Park, H., Seok, C., 2013. GalaxyRefine : protein structure refinement driven by side-chain repacking 41, 384–388. https://doi.org/10.1093/nar/gkt458

Hoover, D.M., Wu, Z., Tucker, K., Lu, W., Lubkowski, J., 2003. Antimicrobial characterization of human β-defensin 3 derivatives. Antimicrob. Agents Chemother. 47. https://doi.org/10.1128/AAC.47.9.2804-2809.2003

Humphrey, W., Dalke, A., Schulten, K., 1996. VMD: Visual molecular dynamics. J. Mol. Graph. 14. https://doi.org/10.1016/0263-7855(96)00018-5

Ikram, A., Zaheer, T., Awan, F.M., Obaid, A., Naz, A., Hanif, R., Paracha, R.Z., Ali, A., Naveed, A.K., Janjua, H.A., 2018. Exploring NS3/4A, NS5A and NS5B proteins to design conserved subunit multi-epitope vaccine against HCV utilizing immunoinformatics approaches. Sci. Rep. 8. https://doi.org/10.1038/s41598-018-34254-5

Jordan, A.B., Blake, D., Beard, J., Beharry, A., Serrette, L., Soleyn, A., Sookhoo, J., Blake, L., Brown, G., Oura, C., 2018. Molecular identification of Eimeria species in broiler chickens in Trinidad, West Indies. Vet. Sci. 5. https://doi.org/10.3390/vetsci5010012

Kaliamurthi, S., Selvaraj, G., Kaushik, A.C., Gu, K.R., Wei, D.Q., 2018. Designing of CD8 + and CD8 + -overlapped CD4 + epitope vaccine by targeting late and early proteins of human papillomavirus. Biol. Targets Ther. 12. https://doi.org/10.2147/BTT.S177901

Kamthania, M., Sharma, D.K., 2015. Screening and structure-based modeling of T-cell

epitopes of Nipah virus proteome: an immunoinformatic approach for designing peptide-based vaccine. 3 Biotech. https://doi.org/10.1007/s13205-015-0303-8

Khatoon, N., Pandey, R.K., Prajapati, V.K., 2017. Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach 1–12. https://doi.org/10.1038/s41598-017-08842-w

Kim, W.H., Chaudhari, A.A., Lillehoj, H.S., 2019. Involvement of T Cell Immunity in Avian Coccidiosis 10, 1–8. https://doi.org/10.3389/fimmu.2019.02732

Kohlgraf, K.G., Pingel, L.C., Dietrich, D.E., Brogden, K.A., 2010. Defensins as anti-inflammatory compounds and mucosal adjuvants. Future Microbiol. https://doi.org/10.2217/fmb.09.104

Larsen, M. V, Lundegaard, C., Lamberth, K., Buus, S., Lund, O., Nielsen, M., 2007. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction 12, 1–12. https://doi.org/10.1186/1471-2105-8-424

Lawal, J.R., Jajere, S.M., Ibrahim, U.I., Geidam, Y.A., Gulani, I.A., Musa, G., Ibekwe, B.U., 2016. Prevalence of coccidiosis among village and exotic breed of chickens in Maiduguri, Nigeria. Vet. World 9. https://doi.org/10.14202/vetworld.2016.653-659

Lillehoj, H.S., 1998. Role of T lymphocytes and cytokines in coccidiosis, in: International Journal for Parasitology. https://doi.org/10.1016/S0020-7519(98)00075-7

Lim, H.X., Lim, J., Jazayeri, S.D., Poppema, S., Poh, C.L., 2021. Development of multi-epitope peptide-based vaccines against SARS-CoV-2. Biomed. J. https://doi.org/10.1016/j.bj.2020.09.005

Meade, K.G., O'Farrelly, C., 2019. B-Defensins: Farming the microbiome for homeostasis and health. Front. Immunol. https://doi.org/10.3389/fimmu.2018.03072

Mohan, T., Sharma, C., Bhat, A.A., Rao, D.N., 2013. Modulation of HIV peptide antigen specific cellular immune response by synthetic α- and β-defensin peptides. Vaccine 31. https://doi.org/10.1016/j.vaccine.2013.01.041

Nain, Z., Karim, M.M., Sen, M.K., Adhikari, U.K., 2019. Structural basis and designing of peptide vaccine using PE-PGRS family protein of mycobacterium ulcerans - An integrated vaccinomics approach. bioRxiv. https://doi.org/10.1101/795146

Narula, A., Kumar, R., Khatoon, N., Mishra, A., Kumar, V., 2018. Excavating chikungunya genome to design B and T cell multi-epitope subunit vaccine using comprehensive immunoinformatics approach to control chikungunya infection Infection , Genetics and Evolution Excavating chikungunya genome to design B and T cell mult. Infect. Genet. Evol. 61, 4–15. https://doi.org/10.1016/j.meegid.2018.03.007

Pandey, R.K., Bhatt, T.K., Prajapati, V.K., 2018a. Novel Immunoinformatics Approaches to Design Multi- epitope Subunit Vaccine for Malaria by Investigating Anopheles Salivary Protein 1–11. https://doi.org/10.1038/s41598-018-19456-1

Pandey, R.K., Bhatt, T.K., Prajapati, V.K., 2018b. Novel Immunoinformatics Approaches to Design Multi- epitope Subunit Vaccine for Malaria by Investigating Anopheles Salivary Protein. Sci. Rep. 1–11. https://doi.org/10.1038/s41598-018-19456-1

Pandey, R.K., Dahiya, S., Mahita, J., Sowdhamini, R., Prajapati, V.K., 2019. Vaccination and immunization strategies to design Aedes aegypti salivary protein based subunit vaccine

tackling Flavivirus infection. Int. J. Biol. Macromol. 122. https://doi.org/10.1016/j.ijbiomac.2018.09.071

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera — A Visualization System for Exploratory Research and Analysis. https://doi.org/10.1002/jcc.20084

Putri, N., Wulandari, I., Ernawati, R., Rantam, F.A., 2019. MULTI-EPITOPE PEPTIDE VACCINE PREDICTION AGAINST NEWCASTLE DISEASE VIRUS USING IMMUNO-INFORMATICS APPROACHES 1, 1–8. https://doi.org/10.15547/bjvm.2019-0074

Raoufi, E., Hemmati, M., Eftekhari, S., Khaksaran, K., Mahmodi, Z., 2020. Epitope Prediction by Novel Immunoinformatics Approach : A State - of - the - art Review. Int. J. Pept. Res. Ther. 26, 1155–1163. https://doi.org/10.1007/s10989-019-09918-z

Reche, P.A., Fernandez-Caldas, E., Flower, D.R., Fridkis-Hareli, M., Hoshino, Y., 2014. Peptide-based immunotherapeutics and vaccines. J. Immunol. Res. https://doi.org/10.1155/2014/256784

Rodr, S.D., Quiroz-castañeda, R.E., Aguilar-d, H., Vara-pastrana, E., Pescador-p, D., Amaro-estrada, I., Mart, F., 2020. Immunoinformatic Analysis to Identify Proteins to Be Used as Potential Targets to Control Bovine Anaplasmosis 2020.

Sormanni, P., Aprile, F.A., Vendruscolo, M., Tessier, P.M., 2015. Rational design of antibodies targeting specific epitopes within intrinsically disordered proteins. Proc. Natl. Acad. Sci. U. S. A. 112. https://doi.org/10.1073/pnas.1422401112

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry Coccidiosis: Design and Interpretation of Vaccine Studies. Front. Vet. Sci. https://doi.org/10.3389/fvets.2020.00101

Tahir, M., Shokat, Z., Muneer, I., Ashfaq, U.A., 2020. Multiepitope-Based Subunit Vaccine Design and Evaluation against Respiratory Syncytial Virus Using Reverse Vaccinology Approach.

Tan, L., Liao, Y., Fan, J., Zhang, Y., Mao, X., Sun, Y., Song, C., Qiu, X., Meng, C., Ding, C., 2016. Prediction and identification of novel IBV S1 protein derived CTL epitopes in chicken. Vaccine 34. https://doi.org/10.1016/j.vaccine.2015.11.042

Tani, K., Murphy, W.J., Chertov, O., Salcedo, R., Koh, C.Y., Utsunomiya, I., Funakoshi, S., Asai, O., Herrmann, S.H., Wang, J.M., Kwak, L.W., Oppenheim, J.J., 2000. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int. Immunol. 12. https://doi.org/10.1093/intimm/12.5.691

Tellez, G., Shivaramaiah, S., Barta, J., Hernandez-Velasco, X., Hargis, B., 2014. Coccidiosis: recent advancements in the immunobiology of Eimeria species, preventive measures, and the importance of vaccination as a control tool against these Apicomplexan parasites. Vet. Med. Res. Reports 23. https://doi.org/10.2147/vmrr.s57839

Thompson, J.D., Gibson, T.J., Higgins, D.G., 2003. Multiple Sequence Alignment Using ClustalW and ClustalX. Curr. Protoc. Bioinforma. 00. https://doi.org/10.1002/0471250953.bi0203s00

Vries, S.J. De, Rey, J., Schindler, C.E.M., Zacharias, M., Tuffery, P., 2017. The

pepATTRACT web server for blind , large-scale peptide – protein docking 45, 361–364. https://doi.org/10.1093/nar/gkx335

Wallach, M., 2010. Role of antibody in immunity and control of chicken coccidiosis. Trends Parasitol. https://doi.org/10.1016/j.pt.2010.04.004

Wang, X., Zhou, D., Wang, G., Huang, L., Zheng, Q., Li, C., Cheng, Z., 2017. A novel multi-variant epitope ensemble vaccine against avian leukosis virus subgroup J. Vaccine 1–6. https://doi.org/10.1016/j.vaccine.2017.10.019

Wiederstein, M., Sippl, M.J., 2007. ProSA-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 35. https://doi.org/10.1093/nar/gkm290

Zhang, H. hua, Yang, X. mei, Xie, Q. mei, Ma, J. yun, Luo, Y. na, Cao, Y. chang, Chen, F., Bi, Y. zuo, 2010. The potent adjuvant effects of chicken β-defensin-1 when genetically fused with infectious bursal disease virus VP2 gene. Vet. Immunol. Immunopathol. 136. https://doi.org/10.1016/j.vetimm.2010.02.018

Zhao, L., Zhang, M., Cong, H., 2013. Advances in the study of HLA-restricted epitope vaccines. Hum. Vaccines Immunother. 9. https://doi.org/10.4161/hv.26088

Zhao, M., Wei, D.Q., 2018. Rare Diseases: Drug Discovery and Informatics Resource. Interdiscip. Sci. Comput. Life Sci. https://doi.org/10.1007/s12539-017-0270-3

Zhou, Z., Wang, Z., Cao, L., Hu, S., Zhang, Z., Qin, B., Guo, Z., Nie, K., 2013. Upregulation of chicken TLR4, TLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with Eimeria tenella in vitro. Exp. Parasitol. 133. https://doi.org/10.1016/j.exppara.2013.01.002

CHAPTER 5

5.0 GENERAL DISCUSSION AND RECOMMENDATION

Using species-specific primers , the prevalence of *E. tenella* (68%) in different broiler chicken farms at Mpumalanga province of South Africa is in accordance with the recently reported high prevalence of *E. tenella* (68.4) in chickens from different farms in KwaZulu-Natal province of South Africa (Fatoba et al., 2020). The presence of *E. tenella* in different poultry farms and free ranges and backyard reared chickens in South Africa and other countries showed that subclinical cases of coccidiosis caused by *E. tenella* are still ongoing at different farms across South Africa and other countries. *Eimeria tenella* is the most infective and one of the most frequent isolated *Eimeria* species implicated in chicken's coccidiosis (Blake et al., 2015), hence it was used in the current study to detect the level of genetic diversity amongst *Eimeria tenella* Apical Membrane Antigen 1(*Et*AMA1) in selected farms in Mpumalanga province and predict vaccine epitopes from this antigen.

The current study indicates that *Et*AMA1 is a good candidate for vaccine development against chicken coccidiosis. The low diversity reported among this antigen is in agreement with the report of Blake *et al*., 2015 which favour its use in future vaccine development (Blake et al., 2015). The *Et*AMA1 sequences of this study and those retrieved from GeneBank were successfully used to construct an antigenic and non-allergen multiepitope vaccine following immunoinformatics approaches. The predicted vaccine showed a strong affinity with TLR4 which is one of the most important innate immune receptors, which plays a crucial part against coccidiosis (Zhou et al., 2013). The predicted multiepitope vaccine, once validated experimentally, could serve as an effective vaccine against chicken coccidiosis worldwide.

Immunoinformatics strategies have been used to identify conserved sequences in different viral proteins such as nucleoprotein (Ali and Islam, 2015) and glycoprotein (Dash et al., 2017), Zika virus glycoprotein, Chikungunya virus proteins (Alam et al., 2016), Nipah Virus fusion and glycoprotein (Ali et al., 2015) nucleocapsid and glycoprotein of Rift Valley fever virus (Adhikari and Rahman, 2017). Multiepitope vaccine against *Mycobacterium ulcerans* has also been designed using integrated advanced vaccinomic methods (Nain et al., 2020). Other studies have also used this technique to design a multiepitope vaccine, which could stimulate specific humoral and cellular immune responses against the 2019 novel coronavirus (SARS-CoV-2) (Naz et al., 2020; Sanami et al., 2020).

The current study has the potential to provide very significant information that can be used to improve poultry farming in South Africa and worldwide. Researchers in the field will benefit from this study and provide relevant information to local farmers and the poultry industry to implement effective control measures against coccidiosis.

5.1 Conclusion

In conclusion, the fight against the impact of coccidiosis in poultry at different farms across South Africa and other countries is an ongoing process. Different strategies have been developed to design, develop, and produce a cost-effective and affordable vaccine against poultry coccidiosis. The absence of diversity reported among *Et*AMA1 in this present study further confirms the reliability of this vaccine candidate, and as such, designing a peptide-based vaccine from this vaccine candidate using an immunoinformatics approach could change the tide in the fight against chicken coccidiosis.

5.2 Limitations and future Recommendations

The research project was indeed a success. However, few limitations were faced during the study, including the lack of published papers on *Et*AMA1 diversity and unavailability of online software for chicken major histocompatibility complex (MHC). The scarcity of published papers on the genetic diversity of AMA1 in *Eimeria* species made it nearly impossible to discuss the results from this study. Also, due to the disparity of result generated by diversity parameters, further studies should be carried out on the diversity of *Et*AMA1 from different provinces of South Africa, using the full length of *Et*AMA1 sequences as previously done by Blake *et al*., 2015. The stability of the constructed multiepitope in this study through molecular dynamics should be carried out.

5.3 References

Abdelli, N., Pérez, J.F., Vilarrasa, E., Luna, I.C., Melo-Duran, D., D'angelo, M., Solà-Oriol, D., 2020. Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge. Animals 10. https://doi.org/10.3390/ani10020259

Adhikari, U.K., Rahman, M.M., 2017. Overlapping CD8 + and CD4 + T-cell epitopes identification for the progression of epitope-based peptide vaccine from nucleocapsid and glycoprotein of emerging Rift Valley fever virus using immunoinformatics approach. Infect. Genet. Evol. 56. https://doi.org/10.1016/j.meegid.2017.10.022

Alam, A., Ali, S., Ahamad, S., Malik, M.Z., Ishrat, R., 2016. From ZikV genome to vaccine: in silico approach for the epitope-based peptide vaccine against Zika virus envelope glycoprotein. Immunology 149. https://doi.org/10.1111/imm.12656

Ali, M.T., Islam, M.O., 2015. A highly conserved GEQYQQLR epitope has been identified in the nucleoprotein of Ebola virus by using an in silico approach. Adv. Bioinformatics 2015. https://doi.org/10.1155/2015/278197

Ali, M.T., Morshed, M.M., Hassan, F., 2015. A Computational Approach for Designing a Universal Epitope-Based Peptide Vaccine Against Nipah Virus. Interdiscip. Sci. Comput. Life Sci. 7. https://doi.org/10.1007/s12539-015-0023-0

Angwech, H., Tavaniello, S., Ongwech, A., Kaaya, A.N., Maiorano, G., 2019. Efficacy of In ovo delivered prebiotics on growth performance, meat quality and gut health of kuroiler chickens in the face of a natural coccidiosis challenge. Animals 9. https://doi.org/10.3390/ani9110876

Bargieri, D.Y., Andenmatten, N., Lagal, V., Thiberge, S., Whitelaw, J.A., Tardieux, I., Meissner, M., Ménard, R., 2013. Apical membrane antigen 1 mediates apicomplexan parasite attachment but is dispensable for host cell invasion. Nat. Commun. 4. https://doi.org/10.1038/ncomms3552

Blake, D.P., Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Jatau, I.D., Ayoade, S., Kawahara, F., Moftah, A., Reid, A.J., Adebambo, A.O., Zapata, R.Á., Rao, A.S.R.S., Thangaraj, K., Banerjee, P.S., Dhinakar-Raj, G., Raman, M., Tomley, F.M., 2015. Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. Proc. Natl. Acad. Sci. U. S. A. 112. https://doi.org/10.1073/pnas.1506468112

Blake, D.P., Pastor-Fernández, I., Nolan, M.J., Tomley, F.M., 2017. Recombinant anticoccidial vaccines - a cup half full? Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2017.10.009

Blake, D.P., Worthing, K., Jenkins, M.C., 2020. Exploring Eimeria genomes to understand population biology: Recent progress and future opportunities. Genes (Basel). https://doi.org/10.3390/genes11091103

Borgonovo, F., Ferrante, V., Grilli, G., Pascuzzo, R., Vantini, S., Guarino, M., 2020. A data-driven prediction method for an early warning of coccidiosis in intensive livestock systems: A preliminary study. Animals 10. https://doi.org/10.3390/ani10040747

Chapman, H.D., Jeffers, T.K., 2014. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. Int. J. Parasitol. Drugs Drug Resist. 4.

https://doi.org/10.1016/j.ijpddr.2014.10.002

Crane, M.S.T.J., Murray, P.K., Mckenzie, M.E., Perry, E., 1986. Immunisation of young broiler chickens with low level infections of eimeria tenella, E. Acer Vulina or E. Maxima. Avian Pathol. 15. https://doi.org/10.1080/03079458608436287

Dash, R., Das, R., Junaid, M., Akash, M.F.C., Islam, A., Hosen, S.M.Z., 2017. In silico-based vaccine design against Ebola virus glycoprotein. Adv. Appl. Bioinforma. Chem. 10. https://doi.org/10.2147/AABC.S115859

Eckford, S., Grace, K., Harris, C., Reeves, H., 2013. UK Veterinary Antibiotic Resistance and Sales Surveillance: UK-VARSS 2013. UK Vet. Antibiot. Resist. Sales Surveill. UK VARSS 132.

Fatoba, A.J., Zishiri, O.T., Blake, D.P., Peters, S.O., Lebepe, J., Mukaratirwa, S., Adeleke, M.A., 2020. Study on the prevalence and genetic diversity of eimeria species from broilers and free-range chickens in kwazulu-natal province, south africa. Onderstepoort J. Vet. Res. 87. https://doi.org/10.4102/ojvr.v87i1.1837

Flower, D.R., 2009. Bioinformatics for Vaccinology, Bioinformatics for Vaccinology. https://doi.org/10.1002/9780470699836

Han, H., Xue, P., Dong, H., Zhu, S., Zhao, Q., Huang, B., 2016. Screening and characterization of apical membrane antigen 1 interacting proteins in Eimeria tenella. Exp. Parasitol. 170. https://doi.org/10.1016/j.exppara.2016.09.014

Haug, A., Gjevre, A.G., Thebo, P., Mattsson, J.G., Kaldhusdal, M., 2008. Coccidial infections in commercial broilers: Epidemiological aspects and comparison of Eimeria species identification by morphometric and polymerase chain reaction techniques. Avian Pathol. 37. https://doi.org/10.1080/03079450801915130

Hinsu, A.T., Thakkar, J.R., Koringa, P.G., Vrba, V., Jakhesara, S.J., Psifidi, A., Guitian, J., Tomley, F.M., Rank, D.N., Raman, M., Joshi, C.G., Blake, D.P., 2018. Illumina Next Generation Sequencing for the Analysis of Eimeria Populations in Commercial Broilers and Indigenous Chickens. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00176

Jiang, L., Lin, J., Han, H., Dong, H., Zhao, Q., Zhu, S., Huang, B., 2012. Identification and characterization of eimeria tenella apical membrane antigen-1 (ama1). PLoS One 7. https://doi.org/10.1371/journal.pone.0041115

Kumar, S., Garg, R., Moftah, A., Clark, E.L., Macdonald, S.E., Chaudhry, A.S., Sparagano, O., Banerjee, P.S., Kundu, K., Tomley, F.M., Blake, D.P., 2014. An optimised protocol for molecular identification of Eimeria from chickens. Vet. Parasitol. 199. https://doi.org/10.1016/j.vetpar.2013.09.026

Li, J., Wang, F., Ma, C., Huang, Y., Wang, D., Ma, D., 2018. Recombinant lactococcus lactis expressing Eimeria tenella AMA1 protein and its immunological effects against homologous challenge. Exp. Parasitol. 191. https://doi.org/10.1016/j.exppara.2018.05.003

Liu, L., Huang, X., Liu, J., Li, W., Ji, Y., Tian, D., Tian, L., Yang, X., Xu, L., Yan, R., Li, X., Song, X., 2017. Identification of common immunodominant antigens of Eimeria tenella, Eimeria acervulina and Eimeria maxima by immunoproteomic analysis. Oncotarget 8. https://doi.org/10.18632/oncotarget.16824

Ma, D., Huang, Y., Ma, C., Zhang, L., Wang, J., Wang, D., Li, J., Dalloul, R.A., 2019.

Eimeria tenella: Specific EtAMA1-binding peptides inhibit sporozoite entry into host cells. Poult. Sci. 98. https://doi.org/10.3382/ps/pez298

María, R.R., Arturo, C.J., Alicia, J.A., Paulina, M.G., Gerardo, A.O., 2017. The Impact of Bioinformatics on Vaccine Design and Development, in: Vaccines. InTech. https://doi.org/10.5772/intechopen.69273

Nain, Z., Karim, M.M., Sen, M.K., Adhikari, U.K., 2020. Structural basis and designing of peptide vaccine using PE-PGRS family protein of Mycobacterium ulcerans—An integrated vaccinomics approach. Mol. Immunol. 120. https://doi.org/10.1016/j.molimm.2020.02.009

Naz, A., Shahid, F., Butt, T.T., Awan, F.M., Ali, A., Malik, A., 2020. Designing Multi-Epitope Vaccines to Combat Emerging Coronavirus Disease 2019 (COVID-19) by Employing Immuno-Informatics Approach. Front. Immunol. 11. https://doi.org/10.3389/fimmu.2020.01663

Oakes, R.D., Kurian, D., Bromley, E., Ward, C., Lal, K., Blake, D.P., Reid, A.J., Pain, A., Sinden, R.E., Wastling, J.M., Tomley, F.M., 2013. The rhoptry proteome of Eimeria tenella sporozoites. Int. J. Parasitol. 43. https://doi.org/10.1016/j.ijpara.2012.10.024

Pastor-Fernández, I., Kim, S., Billington, K., Bumstead, J., Marugán-Hernández, V., Küster, T., Ferguson, D.J.P., Vervelde, L., Blake, D.P., Tomley, F.M., 2018. Development of cross-protective Eimeria-vectored vaccines based on apical membrane antigens. Int. J. Parasitol. 48. https://doi.org/10.1016/j.ijpara.2018.01.003

Peek, H.W., Landman, W.J.M., 2011. Coccidiosis in poultry: Anticoccidial products, vaccines and other prevention strategies. Vet. Q. 31. https://doi.org/10.1080/01652176.2011.605247

Sanami, S., Zandi, M., Pourhossein, B., Mobini, G.R., Safaei, M., Abed, A., Arvejeh, P.M., Chermahini, F.A., Alizadeh, M., 2020. Design of a multi-epitope vaccine against SARS-CoV-2 using immunoinformatics approach. Int. J. Biol. Macromol. 164. https://doi.org/10.1016/j.ijbiomac.2020.07.117

SAPA, 2017. SAPA Broiler Industry Summary on two pages 2017.

Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009. Genomic analysis of eimeria spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. J. Parasitol. 95. https://doi.org/10.1645/GE-1898.1

Shirley, M.W., 2000. The genome of Eimeria spp., with special reference to Eimeria tenella - A coccidium from the chicken. Int. J. Parasitol. 30. https://doi.org/10.1016/S0020-7519(99)00183-6

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry Coccidiosis: Design and Interpretation of Vaccine Studies. Front. Vet. Sci. https://doi.org/10.3389/fvets.2020.00101

Tomley, F., 1997. Techniques for isolation and characterization of apical organelles from Eimeria tenella sporozoites. Methods A Companion to Methods Enzymol. 13. https://doi.org/10.1006/meth.1997.0509

5.4 Supplementary Information

List of Farms where samples were collected

A. Elzo Broilers
B. Tlatsa poto project
C. Arthurseat chicken farm
D. Xumani broilers farm
E. Mabika poultry farm
F. Ermelo Backyard
G. Standarton Backyards
H. Bethal
I. Siseko farm
J. Middleburg

Table S1 : Identical sequences represented by nodes

Node Label	Matching Sequences
EtAMA1_isolate_Mpumalanga_2EL_1F	EtAMA1_isolate_Mpumalanga_3A_1F
	EtAMA1_isolate_Mpumalanga_5A_1F
	EtAMA1_isolate_Mpumalanga_6AM_1F
	EtAMA1_isolate_Mpumalanga_7AM_1F
	EtAMA1_isolate_Mpumalanga_8AM_1F
	EtAMA1_isolate_Mpumalanga_9EL_1F
	EtAMA1_isolate_Mpumalanga_10E_1F
	EtAMA1_isolate_Mpumalanga_10S_1F
	EtAMA1_isolate_Mpumalanga11A_1F
	EtAMA1_isolate_Mpumalanga_11EL_1F
	EtAMA1_isolate_Mpumalanga_11X_1F
	EtAMA1_isolate_Mpumalanga_13S_1F
	EtAMA1_isolate_Mpumalanga_14S_1F
	EtAMA1_isolate_Mpumalanga_15D_1F
	EtAMA1_isolate_Mpumalanga_16X_1F
	EtAMA1_isolate_Mpumalanga_17EL_1F
	EtAMA1_isolate_Mpumalanga_17TP_1F
	EtAMA1_isolate_Mpumalanga_18EL_1F
	EtAMA1_isolate_Mpumalanga_18ER_1F
	EtAMA1_isolate_Mpumalanga_18TP_1F
	EtAMA1_isolate_Mpumalanga_18X_1F
	EtAMA1_isolate_Mpumalanga_19ER_1F
	EtAMA1_isolate_Mpumalanga_20ER_1F
	EtAMA1_isolate_Mpumalanga_20TP_1F
	EtAMA1_isolate_Mpumalanga_21K_1F
	EtAMA1_isolate_Mpumalanga_22ER_1F

	EtAMA1_isolate_Mpumalanga_23ER_1F EtAMA1_isolate_Mpumalanga_23TP_1F EtAMA1_isolate_Mpumalanga_27TP_1F EtAMA1_isolate_Mpumalanga_31K_1F EtAMA1_isolate_Mpumalanga_33K_A1F EtAMA1_isolate_Mpumalanga_K22K_1F
LN610016.1_EtAMA1_isolate_Nigeria2_05	LN610000.1_EtAMA1_isolate_Egypt_01 LN609997.1_EtAMA1_isolate_Libya_3 LN609994.1_EtAMA1_isolate_Germany_01 LN609993.1_EtAMA1_isolate_UK_01 LN609992.1_EtAMA1_isolate_Venezuela_05 LN609987.1_EtAMA1_isolate_India_05 LN609982.1_EtAMA1_isolate_China LN610018.1_EtAMA1_isolate_Nigeria2_07 LN610014.1_EtAMA1_isolate_Nigeria2_03 LN610012.1_EtAMA1_isolate_Nigeria2_01 LN610001.1_EtAMA1_isolate_Egypt_02 LN609996.1_EtAMA1_isolate_Libya_02 LN609989.1_EtAMA1_isolate_Venezuela_02 LN609988.1_EtAMA1_isolate_Venezuela_01 LN609985.1_EtAMA1_isolate_India_01 LN609981.1_EtAMA1_isolate_Nigeria_05 LN609980.1_EtAMA1_isolate_Japan_Nt2 LN609976.1_EtAMA1_isolate_UK_Houghton LN610031.1_EtAMA1_isolate_Nigeria3_10 LN610030.1_EtAMA1_isolate_Nigeria3_09 LN610029.1_EtAMA1_isolate_Nigeria3_08 LN610028.1_EtAMA1_isolate_Nigeria3_07 LN610027.1_EtAMA1_isolate_Nigeria3_06 LN610026.1_EtAMA1_isolate_Nigeria3_05 LN610025.1_EtAMA1_isolate_Nigeria3_04 LN610024.1_EitAMA1_isolate_Nigeria3_03 LN610023.1_EtAMA1_isolate_Nigeria3_02 LN610022.1_EtAMA1_isolate_Nigeria3_01 LN610021.1_EtAMA1_isolate_Nigeria2_10 LN610020.1_EtAMA1_isolate_Nigeria2_09 LN610019.1_EtAMA1_isolate_Nigeria2_08 LN610017.1_EtAMA1_isolate_Nigeria2_06 LN610015.1_EtAMA1_isolate_Nigeria2_04 LN610013.1_EtAMA1_isolate_Nigeria2_02 LN610011.1_EtAMA1_isolate_Nigeria1_10 LN610010.1_EtAMA1_isolate_Nigeria1_09 LN610009.1_EtAMA1_isolate_Nigeria1_08 LN610008.1_EtAMA1_isolate_Nigeria1_07 LN610007.1_EtAMA1_isolate_Nigeria1_06 LN610006.1_EtAMA1_isolate_Nigeria1_05 LN610005.1_EtAMA1_isolate_Nigeria1_04 LN610004.1_EtAMA1_isolate_Nigeria1_03 LN610003.1_EtAMA1_isolate_Nigeria1_02 LN610002.1_EtAMA1_isolate_Nigeria1_01

LN609999.1_EitAMA1_isolate_Libya_05
LN609998.1_EtAMA1_isolate_Libya_04
LN609995.1_EtAMA1_isolate_Libya_01
LN609991.1_EtAMA1_isolate_Venezuela_04
LN609990.1_EtAMA1_isolate_Venezuela_03
LN609986.1_EtAMA1_isolate_India_02
LN609984.1_EtAMA_isolate_India_04
LN609983.1_EtAMA1_isolate_India_03
LN609979.1_EtAMA1_solate_UK_Weybridge
LN609978.1_EtAMA1_isolate_US_Wisconsin
LN609977.1_EtAMA1_isolate_Germany_D