GENETIC CHARACTERIZATION OF ROBUSTNESS AND FITNESS TRAITS IN SOUTH AFRICAN INDIGENOUS CHICKENS

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Preface

The research contained within this dissertation was done by Nokwanda Mtungwa at the University of KwaZulu-Natal (Westville campus) in the discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science, under the supervision of Dr. Oliver Zishiri and Dr. Matthew Adeleke (co-supervisor) from 2015 to 2016. The research was financially supported by a college bursary from the College of Agriculture, Engineering and Science for the first year and the NRF-Freestanding, Innovation and Scarce Skills Masters and Doctoral Scholarship for 2016 for the second year.

This dissertation is mainly based on the Genetic Characterization of Robustness and Fitness Traits in South African Indigenous Chickens, and the reported results are as per investigated candidate gene. None of the text within this thesis was directly taken from previously published or collaborative articles, neither has the contents of this work been submitted to any other institution, except for where the work of others is acknowledged in the text.

We certify that the above information is true.

_____

Dr. Oliver Zishiri (Supervisor)

Date:……

_____

Dr. Matthew Adeleke (Co-Supervisor)

Date:……

DECLARATION OF PLAGIARISM

I, Nokwanda Mtungwa, declare that:

1. This dissertation is my original work, except where indicated not to be.
2. This dissertation has not been submitted to any other institution for any degree or examination.
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DECLARATION OF PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication

Publication 1: (This study will be submitted for peer review)

Mtungwa, N., Zishiri, O.T. and Adeleke, M.A. (2016). The Genetic Characterization of fitness and robustness traits in South African Indigenous Chickens.

Signed……

Abstract

Cytokines play an essential role of promoting both immune and inflammatory responses by managing other cells and tissues. IL-2 aids in the duplication, maturation and differentiation of T- and B-lymphocytes, whilst IFN-γ aids in promoting the functioning of anti-viral and macrophage-activating factor, and potentially increases the expression of MHC class II genes and triggers the release of nitric oxide in macrophages. Many studies have described the role and structure of cytokines in mammals. However, a little information is found about the role and structure of avian cytokines. The main aim of this study was to detect and genotype for single nucleotide polymorphisms of the IL-2 and IFN-γ genes responsible for disease resistance by using the polymerase chain reaction restriction fragment-length polymorphism assay. Three different genotypes were observed in both these loci, AA, AG and GG. Thereafter, population-genetic measurements were compiled and analyzed using the PopGene V. 1.31 software and 'Shane's simple guide to F statistics'. Upon analysis of genetic measurements complete inbreeding (100%) was observed in the PMB and Kwamashu population for the IFN-γ locus, and in Victoria Market for the IL-2 locus. Excessive inbreeding (90%) was experienced by the Kwamashu population for the IL-2 locus. A fair distribution of homozygotes and heterozygotes was observed in the PMB population for the IFN-γ gene, indicating 50% of both outbreeding and inbreeding within the sub-populations of PMB. Greater heterozygosity (52.38%) was demonstrated by IL-2 in PMB, indicating outbreeding with sub-populations of PMB. An overall genetic differentiation (F_{ST}) of 13.9% was observed, indicating low genetic variability and overall excessive inbreeding in PMB, Kwamashu and Victoria Market. Hence, there were high levels of non-random mating within sub-populations. The IL-2 nuclease activity by the *MnlI* RE resulted in a G/G mutation which was once associated with *Salmonella enteritidis* reduction in the liver of broiler and Old Dutch chickens, caecum of broilers, and the spleen tissue organ. Conversely, the IFN-γ digestion by *TasI* resulted in a mutation associated with induced primary and maximum secondary antibody titre colonization in the immune system of chickens. Although indigenous chickens are considered genetically more robust and fit compared to commercial chickens, in the presence of excessive inbreeding amongst sub-populations, complete isolation occurs as a result of non-random mating within sub-populations. For this reason, the population is limited in diverging due to great chances of genetic drift, and other disruptive changes such as, natural selection, inconsistent allele and genotype frequencies, and gene flow.

Keywords: chicken; disease resistance; heterozygosity; homozygosity; IFN-γ; IL-2; inbreeding; indigenous chickens.

Acknowledgements

I quote, ‘I have seen something else under the sun: The race is not to the swift or the battle to the strong, nor does food come to the wise or wealth to the brilliant or favor to the learned; but time and chance happen to them all (Ecclesiastes 9;11 NLT).’ I would like to honor the Man upstairs, God (the Lord Jesus Christ of Nazareth) for teaching me that everything has its own timing, and when chance hits my way it’s not because I’ve been wiser than any other but because He has graced me with the favor to meet with and recognize chance. I would also like to thank my parents (Nomthandazo, Bheki and Thembisile Mtungwa), my spiritual parents (Norma and Themba Mbambo) and church (Family Worship Centre), my siblings (Anelisa, Frank, Nora and Thabiso Khumalo), and my most scientific uncle, Mr. ‘Enzymes’ Sifiso Mtungwa for always carrying me through prayer and reminding me that, everything works together for good for those who love God.

Eight years ago I entered the University of KwaZulu-Natal as a ‘freshman’ student, caught up in both fear and excitement, but most of all I entered with a vision of being a Scientist, and WOW! Who ever knew I’d be standing where I am today (a post-graduate student)? And for this, I’d like to acknowledge the institution with greatest gratitude for making me feel at home and providing me with all the facilities I needed as a student to succeed. It is said, ‘Have confidence in your leaders and submit to their authority, because they keep watch over you as those who must give an account. Do this so that their work will be a joy, not a burden, for that would be of no benefit to you (Hebrews 13;11 NLT).’ Therefore, I would like to say thank you to my supervisor, Dr. Oliver Zishiri and my co-supervisor, Dr. Matthew Adeleke for never giving up on me, even on my worst days as a student. I can never be more thankful for your patience and teachings that have brought me thus far. Please keep up the good work and never grow weary of doing good.

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Last but not least, I would like to thank the College of Agriculture, Engineering and Science and NRF for financially supporting my master’s degree studies, I would not have received such support from anywhere else, thank you.

List of abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cell
BA	*Brucella abortus*
CEF	Chicken Embryo Fibroblasts
CpG	peptidoglycan and bacterial DNA
CST	Colony-Stimulating Factor
CTL	Cytotoxic T cell
CTX	Cholera Toxin
GPCR	G-protein Coupled Receptors
HWE	Hardy-Weinberg Equilibrium
HVT	Herpesvirus of Turkey
Ig	Immunoglobulin
PCR	Polymerase Chain Reaction
IAM	Infinite Allele Model
ICSBP	Interferon Consensus Binding Protein
IL	Interleukin
IFN	Interfron
iNOS	inducible Nitric Oxide Synthase
IRF	Interfron regulatory Factor
JAK	Janus Kinase

LPS	Lipopolysaccharide
LTA	Lipoteichoic
MCP-1	Monocyte Chemo-Attractant Protein 1
MDV	Marek's Disease Virus
MHC	Major Histocompatibility Complex
NK	Natural Killer
NO	Nitric Oxide
NOD	Nucleotide Binding Oligomerization Domain Proteins
PAMPS	Pathogen associated molecular patterns
PRRs	Pathogen Recognition Receptors
RADP	Random Amplified Polymorphic DNA
RFLP	Restriction-Fragment Length Polymorphisms
RSV	Rous Sarcoma Virus
SE	*Salmonella enteritidis*
SFV	Semiliki Forest Virus
SNPs	Single Nucleotide Polymorphisms
SRBC	Sheep Red Blood Cells
SSM	Stepwise Mutation Model
STAT	Signal Transducer Activator of Transcription
TBE	Tris-borate- EDTA buffer
TCR	T cell Receptor
TLR	Toll-like Receptor

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CHAPTER 1

GENERAL INTRODUCTION

The four main native breeds of South African indigenous chickens include, the Potchefstroom Koekoek, Venda, Ovambo and Nacked Neck (Grobbelaar, Sutherland et al. 2010). However, it is the Potchefstroom Koekoek that is most acknowledged for its meat. South African indigenous chickens are considered most robust and fit compared to commercial chickens because of their ability to live and survive in extreme weather conditions (heat, droughts, and cold), survive low-feed or no food provision, can scavenge for food, and survive on trees whether they are sheltered or not (Nhleko, Slippers et al. 2003). Most small-holder farmers in South Africa keep indigenous chickens for poultry production (eggs and meat) and/or income to sustain the living of their families. However, for flow of income and stability, the farmer must ensure to keep indigenous chickens that genetically have the ability to produce their own eggs, are broody, can generate the right amount of meat and eggs whether in an extensive or semi-intensive poultry production system (Nhleko, Slippers et al. 2003). Egg production is possible through the commercial, semi-intensive and household egg production systems. However, the commercial system is famous for greater quantities of egg produce (>280 eggs/hen in a production cycle). Although indigenous chickens are regarded more environmentally robust and fit, they are not as good egg producers as commercial breeds. The production performance of indigenous chickens can be increased by well-managed feed (e.g. commercial feed diets) (Nhleko, Slippers et al. 2003). For instance, Ethiopian indigenous chickens subjected to an extensive system can produce a range of 40 to 99 eggs annually (Yami 1995), whilst Nigerian indigenous chickens given commercial feed could produce 80 to 90 eggs within less than ten months (Adetayo and Babafunso 2001). It is essential for farmers to know which breed is capable of producing a sufficient amount of eggs under semi-intensive conditions so that the farmer may know which breeds to keep.

Chickens play a highly important role in the study of immunology as they act as the best models of study. Poultry is the world's largest source of meat and egg production for human diets. The poultry industry is responsible for 40% of animal product produce (Lamont, Kaiser et al. 2002). The poultry sector is of high economic importance due to the ability of chickens to produce favorable amounts of meat under less supply of water, energy and low greenhouse gas creation paralleled to various production systems (De Verdal, Narcy et al. 2011). Approximately 80 billion chickens are hatched and culled to produce 105 million tons of meat and 70 million tons of eggs on a yearly basis (Lowenthal, Bean et al. 2013). The

poultry industry will continue to grow worldwide with the persisting human growth rate, daily consumption rate, urbanization and the purchasing demands of eggs and meat by humans (USDA, 2012).

Although chickens have become the world's key protein source for most human diets, they are vulnerable to bacterial, fungal, protozoan and viral pathogens (Stewart, Keyburn et al. 2013). This is a serious threat to human health as these pathogens are transmissible. Infectious diseases are the cause behind the increased level of fatalities in the poultry industry worldwide (Cavero, Schmutz et al. 2009), and not only are they a welfare problem but are major contributors to poultry industry economic losses. For instance, an infection called Colibacillosis has been reported to cause severe growth retardation, suppressed feed intake, uniformity reduction, and several deaths (Ask, van der Waaij et al. 2006). It has also been reported that most food contamination is due to the transmission of Salmonella and Campylobacter (Stewart, Keyburn et al. 2013). Salmonella and Campylobacter are responsible for most food poisoning, hospitalizations, deaths and human illnesses (Mead, Slutsker et al. 1999). Moreover, great economic losses have been witnessed due to the spread of pathogenic viruses. For instance, avian influenza subtype H5N1 has previously caused over 370 human death cases (USDA, 2012). In addition to zoonotic pathogens, there are number of viruses responsible for 100% death cases, such as fowl cholera, fowl pox, infectious bronchitis, infectious bursal disease virus, necrotic enteritis, Marek's Disease, coccidiosis and infectious laryngotracheitis.

The most common control measures used in fighting off avian diseases usually include antibiotics and vaccines. However, less and less antibiotic application will be used in poultry due to consumer demands and regulations. Vaccines on the other hand assist in combating avian infection; however in some cases are not as good combaters due to the diversity of existent serotypes in outbreaks. For instance, vaccination of Colibacillosis is only potent to homologous strains, but is not as effective to heterologous strains (Dho-Moulin and Fairbrother 1999).

It is highly important to understand the genetic basis behind the control of immune responses to disease in order to maintain healthy poultry, especially for human diet. Understanding the genetics of a chicken or a flock is genetically imperative in improving disease resistance because it helps in the identification of the maximal degree to which birds can confer resistance to disease. The major histocompatibility complex (MHC) gene family impact on disease resistance has been investigated due to this complex being highly associated with the robustness and fitness of avian hosts by demonstrating significantly effective immune responses to pathogens (Lamont 1998). In addition, there are also a number of candidate genes that have also demonstrated disease-resistance potential, such as cytokine genes, CD-encoding genes, T cell receptor genes, Nramp1, growth hormone, and the Immunoglobulin genes. However, resistance is usually controlled by polygenes; thus a polygenic phenomenon. Both physiological and molecular genetic

strategies can be employed to produce divergent genetic lines for immuno-competence traits or disease resistance, and have been crossed to produce appropriate populations for mapping molecular markers for desirable performance (Lamont 1998).

The use of genetic approaches in controlling disease and production enhancement is very important and rewarding. The impact of vaccines together with disease resistance can be greatly enhanced through the genetic enhancement of more robust immune responses. Thus, eradication of antibiotic traces in food. Moreover, genetic markers can be employed to direct selection on disease traits. It is generally thought that using well-characterized genetic markers associated with desirable robustness and fitness traits would be more effective and cost-efficient when compared to the welfare problem of extensive pathogen challenge testing which remains both unsafe and expensive. At present, less is known about the actual genes governing resistance traits. Therefore, restricts the broad use of molecular genetic strategies to maintain healthy animals (Lamont, Kaiser et al. 2002).

More research should focus on the impact of genetics on robustness and fitness traits of chicken by detecting non-MHC genetic control of resistance and through additional study of the mechanisms responsible for controlling the expression of genes that confer immune responses.

1.1 Problem Identification

Salmonella is a non-typhoidal Gram negative bacterium which is also known as a contributory agent of Salmonellosis. Salmonellosis stands the world's most mutual food-borne disease and it can be transmitted either in a vertical or a lateral manner. This kind of transmission can occur through both direct and indirect contact with pets, veterinary clinics, zoological gardens, farms, and any other public, private or professional setting (Hoelzer, Moreno Switt et al. 2011). *Salmonella* can be transmitted through environmental contamination and indirect transmission in water and poultry, and this form of transmission can interfere with the control measures of the infection (Hoelzer, Moreno Switt et al. 2011). Poultry products are known as the chief source of *Salmonella enteritidis* (*S. enteritidis*) contamination for humans.

The Salmonellosis disease accounts for 155000 deaths out of 93.8 million outbreaks of the disease that occur on a yearly basis, ranging from 61768000 (5^{th} percentile) to 131634000 (95^{th} percentile). It is estimated that 86% (80.3 million) of these disease outbreaks are food-borne (Majowicz, Musto et al. 2010). In the USA Salmonellosis has caused 10% of food-borne illnesses, 26% of patient admissions in

hospitals, and 31% of mortality cases. And because of these statistics, *Salmonella* has been identified as the key bacterial pathogen for annual hospitalizations and deaths, whilst on the other hand has been ranked second for human illnesses after Campylobacter (Mead, Slutsker et al. 1999).

The approach of multi-drug resistance has been used to govern the transmission of *Salmonella enterica Serovars* from food to humans mutually in industrialized and undeveloped countries, together with pharmaceutical approaches such as, vaccinations and antibiotic treatment. However, antibiotics and vaccines can only be used as temporal control measures, because the consistent traces of antibiotics being found in chicken and its products (egg and meat) is problematic. Moreover, a great number of bacteria have or still are developing resistance to antibiotic and vaccine control measures, which highlights the importance of new control approaches. Lamont suggests that improvement of drug resistance can only be a success due to the polygenic nature of disease resistance (Lamont 1998). Additional reasons that contribute to the ineffective ability of pharmaceutical measures to control transmission or the presence of this disease are as follows: the virulent phenomenon of pathogens, inability to eliminate pathogens in commercial operations, and the concentration of poultry in larger production units (Vint 1997).

Amongst many candidate genes that have been selected for investigation to improve resistance to disease, only a few have been proven capable of reducing the burden of *S. enteritidis* (SE) in chickens. For instance, (Tohidi, Idris et al. 2012) investigated the effect of candidate gene polymorphisms on resistance to SE in Malaysian indigenous chickens, and found a significant relationship of nucleotide substitution in the interleukin-2 (IL-2) gene with SE loads in the caecum and spleen tissue organs. The IL-2 cytokine proves to have important immunological purpose in the immune system by promoting the proliferation of T cells. Interferon gamma (IFN-γ) is also a type of cytokine that influences immunity against SE in chickens by facilitating the up-regulation of pathogen identification and presentation to pathogen recognition receptors (PRRs), and macrophage activation, and expression of the MHC class I complex (Schroder, Hertzog et al. 2004). Zhou, Li et al. (2003) reported the significant effect of the IFN-γ-LF heterozygote genotype on primary antibody response to sheep red blood cells (SRBC).

It has also been reported that the SE bacterial burden found in layer hens can disturb the egg production by reducing it. Gast and Holt (1998) have demonstrated that SE counts in chickens can reduce egg production, and providing control measures such as vaccination and antibiotic use, and competitive exclusion can reduce SE thus its reduction in contaminated eggs.

1.2 Rationale of study

Most domesticated chickens in South Africa are raised by smallholder farmers under low-input systems, and the conservation of these chickens has been encouraged due to them being essential genetic resources, possessing unique genotypes, being under production intimidations, and their replacement with commercial hybrids (Muchadeyi, Sibanda et al. 2005; Muchadeyi, Wollny et al. 2007). The genetic characterization of robustness and fitness traits will aid as an important requirement for the identification and effective supervision and exploitation of South African indigenous chickens, thus their conservation. The conservation flocks of indigenous chickens consist of four chicken breeds, the Venda, the Ovambo, the Naked Neck, and the Potchefstroom koekoek chickens (Van Marle-Koster and Nel 2000). It is believed that the conservation of these four native breeds of chicken could resourcefully reserve genetic diversity contained in the rural area chickens of South Africa (Mtileni, Muchadeyi et al. 2011). A numerous amount of indigenous chicken breeds is potentially prone to extinction if not already extinct. For instance, in Thailand the Red Jungle Fowl is now only found in forested hills or within national parks (Akaboot, Duangjinda et al. 2012). Disease outbreaks also pose serious threats in human diets, especially in developing countries where not only malnutrition is a problem, yet humans are in desperate need of quality protein (Mengesha 2012). According to natural selection, since indigenous chickens can survive in harsh environmental pressures, with low-feed intake and poor management (Farrell 2000), they are considered more robust and fit towards diseases, thus disease resistant (Minga, Msoffe et al. 2004). Therefore, the characterization of indigenous chicken genetic resources is designed for the identification and effective supervision and use of South African indigenous chicken, thus conservation of domestic chickens. Several of indigenous chicken institutions have developed conservation programs, such as the Fowl for Africa Project initiated by the Agricultural Research Council (Van Marle-Koster and Nel 2000).

South African research has also identified the vital socioeconomic roles of village chickens in underprivileged rural areas such as, their ability to convert any household foods to highly nutritious and high quality food, and famously bought food (Mtileni, Muchadeyi et al. 2009). Most rural communities have a preference for village chicken meat and eggs due to them being more lean and tastier. Domesticated chickens are also used by rural communities for traditional purposes, such as rituals and payments of damages amongst families (Mtileni, Muchadeyi et al. 2009). Therefore, conservation of these exceedingly appreciated genetic resources is essential due to the chicken's cultural-historical value, its association to local traditions, their adaptive features, socioeconomic values of increasing rural community income, and their traits of scientific interest.

Although domesticated chickens have been immensely used for poultry production, their genetic potential has not been studied in depth. Only a few studies have exploited the link of the IL-2 and IFNy gene genetic polymorphisms with immune responses in indigenous chickens. South African indigenous chickens have been selected in the current study because microsatellite research has shown that domesticated chickens could have originated from multiple domestication events, resulting in their great measure of genetic diversity (Mtileni, Muchadeyi et al. 2011).

1.3 Aim of the study

- To detect and genotype for single nucleotide polymorphisms of the IL-2 and IFN-γ genes responsible for disease resistance; using the PCR-RFLP method in which lengthy DNA fragments are cut into shorter fragments by the *MnlI* and *TasI* restriction enzymes at their restriction sites after nuclease activity.

Objectives

- To extract Genomic DNA from the collected blood samples using the phenol-chloroform method which involves the phenol-chloroform phase which allows the separation of the phenol and aqueous phase, centrifugation, and de-salting of the concentrated DNA via ethanol precipitation.

- To amplify Genomic DNA using specific primer sets of the IL-2 and IFN-y candidate genes in a PCR process of denaturation, annealing and extension at a 25-40X cycle.

- To determine population-genetic measurements (the alleles, allele frequencies, Hardy-Weinberg Equilibrium, Shannon Index, Chi-square, probability, observed and expected homozygosity (Nei) and heterozygosity) in order,

- To identify the genetic relationship and genetic variation that exists amongst the three different chicken populations in Kwazulu-Natal.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review will briefly discuss the origins and domestication of the different native breeds of the *Gallus gallus domesticus*, and how they are prone to extinction if not already extinct due to multiple reasoning. The genetic characterization of these breeds will also be discussed for proper supervision and use of South African indigenous chickens, thus their preservation. Cytokine genes will also be discussed due to them being potential candidate genes for use in selection programs for increasing genetic resistance against particular disease in indigenous chickens. Previous publications have highlighted the significant association of cytokine genes with reduced bacterial loads in different tissue organs of avian species (Tohidi, Idris et al. 2012), and to achieve this we need to assess the genetic polymorphisms and genetic variation of each potential gene amongst different populations within South Africa, hence the motivation of this current study. The PCR-RFLP method was one of the genetic diversity tools that was used amongst a few others that are discussed, that will help to determine the genetic polymorphisms and genetic variations of these potential candidate genes. Numerous studies have exploited the genetic potential of commercial chickens. However, limited research has exploited the genetic potential and link of cytokine genes genetic polymorphisms with disease resistance in South African indigenous chickens.

2.2 Domestication of the *Gallus gallus*

Amongst all domestic animals, the domestic chicken (*Gallus gallus domesticus*) is recognized as the utmost important and prevalent of animal species worldwide. Domestic chicken is an important animal protein source in human diets. The world's chicken population is approximately 16.2 billion (Badhaso 2012) and approximately 49.3 million of this population is from Ethiopia with 97.3%, 2.32% and 0.38% being indigenous, exotic, and hybrid chickens respectively, manufacturing 72300 metric tons of chicken meat and 78000 of eggs (CSA, 2011). Originally, the Red Jungle Fowl (*Gallus gallus*) comes from the Southeast of Asia (Oka, Ino et al. 2007; FuMHITO, Miyake et al. 1994; Hillel, Groenen et al. 2003) and is believed to be the common ancestor of all domestic animals, however it could also be a result of

multiple origins. Chicken domestication is dated back to 3000 years ago, however archeological remains as old as 8000 years have been found in the Northeast of China (Yellow River) and the Indus Valley in Pakistan, suggesting domestication of chickens to be as ancient as this discovery. Through the years, the wild type chicken has evolved to populations of broilers, layers, bantams, game and fancy breeds and indigenous village chickens. Humans have previously used domestic chickens for entertainment such as gamecock-fighting (Akaboot, Duangjinda et al. 2012), as pets (e.g. Silkie or bantams), and for religious and/or traditional purposes (Alemayhu 2003). It is only around the 20th century that indigenous chickens were used for human consumption. Generally, indigenous chickens are more preferred in human diets compared to other exotic forms due to them having high quality meat and more lean meat (Teltathum and Mekchay 2009), which is tastier and is with less triglycerides and cholesterol (Jaturasitha, Srikanchai et al. 2008). In comparison to domestic chickens, commercial line chickens are advantageous in size and egg production. Therefore, developing chicken lines with the above mentioned desirable traits would be wise.

2.3 Genetic Diversity of the *Gallus gallus*

Originally, domestic chickens had a rich diversity which would have been useful for breeding animals that easily adapt to different weather changes to satisfy both the consumer and farmer strains in the upcoming events. Nonetheless due to intensive selection and breeding for commercial reasons the diversity has been hampered with (Dorji, Daungjinda et al. 2011). Industrialization and globalization of chicken production are also contributing factors to the affected diversity of chicken with less global distribution of chicken genetic resources, thus a restricted breed composition to commercial stocks of broilers and egg layer chickens.

2.4 Genetic characterization of the *Gallus gallus*

Most indigenous chickens are preserved by small-holder farmers with petite incomes in South Africa and most African countries (Mtileni, Muchadeyi et al. 2011). Indigenous chickens harbor vital genetic resources that need to be well-looked-after alongside production extortions and replacement with commercial hybrids (Muchadeyi, Sibanda et al. 2005) (Muchadeyi, Wollny et al. 2007).

The use of microsatellite markers has helped scientists in defining the genetic diversity of chickens within and between chicken populations (Weigend and Romanov 2001). Species variation of modern domesticated chicken species has been identified by using the within genetic diversity measures (Caballero and Toro 2002), whilst the level of divergence was determined by using the between

populations measure. The within population measures assist in revealing the genetic make-up similarity of domestic chickens with the recent chicken populations whilst the between population measure estimates how genetically related populations are. (Eding and Meuwissen 2001) used marker estimated Kinship coefficients amongst populations founded on similarities of marker alleles. These Kinship estimates demonstrated the degree of co-ancestry existing between the well-kept and village chicken populations in South Africa.

The identification and analysis of genetic polymorphisms of candidate genes within the loci that demonstrate robustness and fitness traits can be useful for research based on genetic resistance (i.e. disease resistance).

2.5 Genetic diversity tools

2.5.1 Microsatellites

Phylogenetic relatedness amongst closely related species are normally hindered by the privation of variation. It is believed that using rapidly evolving characters such as microsatellites would create ease in approximating relationships among closely related taxa or within a species. Hence, in 1994 Bowcock suggested the use of microsatellites in analyzing relationships among closely related species and among subpopulations of a single species. Moreover, microsatellites were adopted on the basis of them having high mutation rates (with a minimal high mutation rate of 10^{-4}, Levinson and Gutman, 1987), thus proposing that they are highly informative genetic markers (Bowcock, Ruiz-Linares et al. 1994).

Microsatellite markers are repeated motifs or simple sequence repeats that consist of core sequences of di-, tri-, or tetra-nucleotide units, which are broadly spread across an entire animal genome. These markers are highly polymorphic in nature, and due to this, are an effective tool for identifying genetic variation in closely related breeds or lines of farm animals (Tadano, Kinoshita et al. 2014). The two mechanisms that can elaborate on the polymorphic nature of microsatellite markers are the unequal crossing over among DNA molecules during meiosis and the DNA slippage duplication (Levinson and Gutman 1987). Microsatellites have remained successful in analyzing the variation of population's structure of chickens (Vanhala, Tuiskula-Haavisto et al. 1998; Hillel, Groenen et al. 2003). Microsatellite markers are also known as co-dominant markers, and approximately 651 of these markers have been mapped on the chicken genome (Dodgson et al. 1998). These co-dominant markers have been highly used for molecular genetic assessment and mapping of chickens (Khutib et al. 1993; Cheng, 1997), paternity analysis, individual identification, genetic relatedness among species (Takahashi, Nirasawa et al. 1998),

phylogenetic studies, measuring inbreeding and any differences that exist among populations. Microsatellites usually evolve at fast rates in order to establish relationships of closely related species but their high mutational rates disqualify them for analyzing deep phylogeny which would permit greater degrees of homoplasy in shorter periods. Only a few studies have focused on using microsatellite primers. Thus, (CROOIJMANS, van Oers et al. 1996) and (Vanhala, Tuiskula-Haavisto et al. 1998) used microsatellite primers derived from either white Leghorn or database sequences to approximate allele frequencies in broiler lines (5.2 and 5.7, respectively). Furthermore, Kaiser and Yonash (2000) demonstrated on a genome-wide level that the Red Jungle Fowl (RJF) and the White Leghorn derived microsatellite primers from the US Poultry Genome Mapping kits 1 and 2 are very convenient and highly informative as genetic markers in assessment of broiler populations. Moreover, proved the use of microsatellites as being tremendously capable at amplifying highly polymorphic PCR product both in and amongst broiler populations.

Microsatellite markers originate from both coding and non-coding regions, this allocation enhances the expression of these markers by suppressing the influential effects of selection and environmental pressure. These markers are less than a 100 base pairs (bp) long, and they possess unique DNA sequences which can be amplified via polymerase chain (PCR) reactions. The advantage of these reactions is that their PCR products can be run simultaneously on one gel although derived from different markers. The method also involves less labour, is cost-efficient, and is not time-consuming with minimal cross-contamination. All these advantages make microsatellite markers appropriate genetic markers for estimating genetic diversity (Baumung, Simianer et al. 2004).

Microsatellite markers have high mutational rates due to their highly polymorphic nature; this has been monitored via *in vivo* and *in vitro*. This mutational rate ranges from 10^3 to 10^6. The nature, length and base composition of the repeat motifs of these markers can interfere with the mutational rate and degree at which allelic variation occurs. Longer repeat motifs result in higher mutational rates compared to shorter motifs; however, this increase is not stable. For instance, Scribner and Pearce (2000) reported higher mutational rates for tetra-nucleotides repeat motifs compared to di-nucleotides repeat motifs. They also reported higher mutational rates for sequences enriched with AT bases compare to sequences enriched with GC bases.

There many types of genetic markers besides microsatellites, including minisatellites, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and markers based on the chicken repeat element CR1. The above mentioned genetic markers all individually contribute to the major class of Type II markers that are produced in different manners. All of these markers are genotyped

using PCR which enhances the ability to rapidly score numerous individuals (Cheng 1997), and all these markers have advantageous and disadvantageous roles as molecular tools.

Microsatellite sequences are flanked with PCR primers which aid in amplifying products of lengthy polymorphisms between individuals. Automated DNA sequences help in scoring these lengthy polymorphisms when labeled with fluorescing PCR primers. The ability to automate the genotyping together with the increased probability of finding a DNA polymorphism is the reason why microsatellite markers have been used as standard molecular markers in genetic mapping and genome-wide quantitative trait loci (QTL) searches. More than 320 of these microsatellite markers have been placed on the East Lansing (EL) chicken genetic map (Khatib, Genislav et al. 1993) and (Cheng and Crittenden 1994). The disadvantage of using microsatellite markers lies on the intensive labor needed to design them. For instance, microsatellite-containing clones need to be identified and sequenced, followed by the production of PCR primers, and then polymorphisms can be identified and mapped. Furthermore, it is difficult to find proficient microsatellite markers since those found within the chicken genome exist in smaller sizes and are less frequent than those of human genomes.

Ruyter-Spire (1996) screened and mapped cDNA that remain associated to microsatellite sequences by observing sixty different cDNA with a $(TG)_n$ microsatellite derived from 21000 phage clones. Of all these phage clones 29 primers were designed and 21 demonstrated polymorphisms. Finally, 15 markers were mapped in either the EL or C (Crompton reference family) maps. The advantage of this strategy was that highly polymorphic markers and genes can be mapped concurrently, which in turn assists in developing both the genetic and the comparative maps (Ruyter-Spira, Crooijmans et al. 1996).

2.5.2 Minisatellites

In conjunction to microsatellites, minisatellites also have tandem repeats and a highly polymorphic nature, but differ in having a repeat unit of 10 to 60 bases. The disadvantage of using minisatellites as genetic markers is that they use Southern Hybridization to identify length variation and this method largely prohibits the rate of genotyping individuals. In addition, it is very difficult to distinguish fragments of similar sizes if they are allelic or not. Moreover, minisatellites are not randomly spread across the chicken genome (Cheng 1997). Hence, minisatellites are good markers in defining genetic diversity between individuals and assemblies, but are not good genetic markers for genetic mapping.

2.5.3 Random Amplified Polymorphic DNA (RAPD) Markers

The RAPD markers are polymorphic DNA fragments generated during amplification using a single 10-base oligonucleotide primer. The advantage of using RAPD markers is their dominant ability of detecting the existence or non-existence of a particular allele. However, they are incapable of distinguishing between a homozygous (one copy) individual and a heterozygous (two copy) individual for the allele. Although a great amount of RAPD markers have been mapped, their disadvantage is that they cannot be transferred to other populations and they possess reproducibility problems, thus a downfall for QTL searches (Cheng 1997).

2.5.4 Evolution of microsatellites

There are two mutational models that have been developed for understanding the evolution of microsatellites: (i) the stepwise mutation model (SSM) and (ii) the infinite allele model. The SSM mutational model predicts that for every mutational occurrence an allele will either mutate up by an addition or down by a deletion of a repeat motif (Goldstein, Linares et al. 1995), and the IAM model predicts that a new private allele will be formed for each mutational occurrence that occurs, although one mutational event can occur at each locus. Shriver and Jin (1993) reported that higher mutational rates exist more commonly in microsatellite markers than other known markers, and this is highly unlikely to happen in minisatellites. Thus, the mutational model that allows for homoplasy is the appropriate model for explaining the polymorphisms existing on microsatellite markers. Homoplasy is a mutational event which occurs by chance on one position, involving two individuals descended from different ancestors that will form into a single allele. The SSM model enables homoplasy but fails to give precise measures of genetic variation and genetic distances between populations. The IAM model demonstrates the lowest degree of homoplasy and greater microsatellite evolution; therefore it is the most suitable and preferred for population studies (Estoup, Tailliez et al. 1995).

2.5.5 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) stand as single DNA base changes or single base mutations which are widely spread throughout the genome, they can be found in non-coding regions of a gene (as ncSNP), within coding regions of a gene (as cSNP), in regulatory regions (as rSNP) or in between genes as intronic genes. Although they can exist as bi-, tri-, or tetra-allelic polymorphisms they mainly occur as bi-allelic forms. SNPs occur at a rate of one SNP per 225bp in chickens, which is five times as frequent as that of humans (Vignal, Milan et al. 2002). The SNP is a potential molecular marker used to explore the genetic diversity of plant and farm animal species. Approximately 3.3 million SNPs have been acknowledged in chickens by comparing broilers, layers and the Chinese Silkie and a single wild RJF (Hillel, Granevitze et al. 2007). Most genetic diseases are a result of single base mutations, thus used as promising molecular markers. They are also used to recognize disease resistant genes in chickens (Malek and Lamont 2003). SNPs act as substitutes of microsatellite markers in several associated studies, and aid in gene mapping, defining population structure, and carrying out functional studies.

Although microsatellite markers are famous for their high polymorphic nature (Hillel, Granevitze et al. 2007), SNPs can be more advantageous as genetic markers. SNPs have better coverage over the genome, provide more precise predictions of genetic relatedness, have small levels of spontaneous mutation, more efficient and cost-effective genetic tools, and are more stable than RFLP and microsatellites. However, their bi-allelic nature makes them less informative.

This current study will detect and genotype for SNPs of the interleukin-2 and interferon-gamma candidate genes responsible for disease resistance using the polymerase chain reaction restriction fragment-length polymorphisms assay.

2.6 The Avian immune system

2.6.1 The Innate immune system

There are two pathways that chickens engage themselves into for their protection against infectious disease. This is through the modulation of the innate immune system, and that of the adaptive immune system. The innate immune system is the first line of defense against foreign microbes at the site of infection. The chief mechanisms of the innate immune system are physical epithelial and chemical barriers (such as skin, feathers and mucus synthesis), phagocytic leukocytes, dendritic cells, Natural Killer (NK) cells, circulating plasma proteins and cytokines. This immune system mediates immune responses against viral infections by modulating and reducing viral replication and transmission. During the initial phases of the infection the host utilizes germ-line encoded receptors. These receptors are called Pathogen Recognition Receptors (PRRs) and they are responsible for recognizing Pathogen Associated Molecular Patterns (PAMPs) of infectious microbes. The PAMP-PRR bond promotes the activation of intracellular signals that will activate genes responsible for encoding the action of pro-inflammatory cytokines, anti-apoptotic factors, together with antimicrobial peptides (Paul, Brisbin et al. 2013). The host also fights infections by mediating immune responses through the production of antimicrobial peptides, cytokines (Interleukin – IL) and chemokine's (Interferon – IFN) (Mogensen 2009). This is achieved by the adoption of TLRs and nucleotide binding oligomerization domain proteins (NODs) to target the virus and synthesize necessary signalling pathways and activation of the transcription factor for interferon and cytokine release (Paul, Brisbin et al. 2013). The innate immune system provides a more broadly defined and quick defence against pathogens compared to the adaptive immune system.

2.6.2 Innate immunity

The most effective innate host responses are mediated upon recognition of viral nucleic acids by TLRs (Veronica et al 2006). This interaction promotes the production of type 1 IFNs which possess a very vital antiviral action against infection. The antiviral activity of type 1 IFNs involves the use of interferon regulatory factors (IRFs), and a family of transcription factors which aid in antiviral defense, cell growth and the regulation of the immune system. Karaca, Anobile et al. (2004) and Morgan, Sofer et al. (2001) demonstrated an up-regulation of IRFs, particularly IRF-1 and IRF-3 in chicken embryo fibroblasts (CEF) challenged with either MDV or herpesvirus of turkey (HVT). Moreover, Quere, Tivas et al. (2005) also

demonstrated a stimulated expression of IFN-α in MDV challenged susceptible chicken. These studies prove the imperative immunological role of IFNs in immunity against MD (Quere, Rivas et al. 2005).

Several studies have demonstrated the *in vitro* and *in vivo* role of macrophages in MDV pathogenesis and immunity against MD ((Barrow, Burgess et al. 2003; Djeraba, Bernardet et al. 2000). Macrophages are phagocytic cells that act in the first line of defense against infections, and aid in the destruction of damaged cells and dead cells within the host. Upon MDV infection macrophages function to either engulf MDV challenged cells by phagocytosis or terminate viral replication of MD (Barrow, Burgess et al. 2003; Djeraba, Bernardet et al. 2000). Macrophages are also vital for the production of pro-inflammatory cytokines and the expression of their antiviral activities. After infection, macrophages are activated to stimulate the synthesis of nitric oxide (NO), which is essential for suppression of MDV duplication within the host (Djeraba, Musset et al. 2002; Xing and Schat 2000). A higher level of expression of inducible nitric oxide synthase (iNOS) upon infection has been reported in tissues such as, the spleen, brain and lungs of challenged chickens (Xing and Schat 2000, Abdul-Careem, Haq et al. 2009). Moreover, these studies have shown that an enhanced secretion of NO is associated with greater suppression of MDV replication.

Natural Killer cells are responsible for expressing powerful apoptotic antiviral activities against virally infected cells or cancerous cells, through the ligation of apoptotic receptors (Fas and FasL) and the synthesis of granular substances. These substances include serine proteases called granzymes and pore-forming perforins. Birds challenged with MDV have demonstrated an up-regulation of granzyme A, NK-lysin, and perforin as immunity against the MDV infection. More studies report the ability of NK cells to confer potent activity against MDV even in MDV resistant and vaccinated chickens in contrast to MDV-susceptible and unvaccinated chickens (Garcia-Camacho, Schat et al. 2003; Heller and Schat 1987). Quere and Dambrine (1988) also reported the highly effective and potent immune responses NK cells demonstrate against infected MDV cells by interfering with the transformation stages of the MDV cycle. Hence, this information highlights the important function of NK cells against host infections such as MDV and their antiviral activity during the primary cytolytic stages of the infection.

2.6.3 The Adaptive immune system

The adaptive immune system is also branded as the acquired immune system or the second line of defense as it comprises of memory cells that enable the host to mediate a more powerful response in cases of re-introduction to the same pathogen. The adaptive immune system uses the subtypes of T- and B-lymphocytes in mediating an Antigen (Ag)-specific immune response (Medzhitov and Janeway 1997). The B-lymphocytes are liable for mediating a humoral immune response through the making of antibodies (Abs), and T-lymphocytes mediate cell-mediated immunity (Le Bon and Tough 2002). T-cell-mediated immunity is conferred when $CD4^+$ and $CD8^+$ T-cell receptors bond with MDV antigens of the major histocompatibility complex (MHC) molecules. This immune system has been reported for its imperative antiviral activities against the herpesvirus, and the release of antibodies against many MDV proteins. The adaptive immune system provides a more pathogen specific response which is much slower compared to the innate immune system.

2.6.4 Humoral antibody immune response

Antibodies provide humoral immunity through the process of neutralization, opsonization and complement activation. In neutralization, bacterial adherence is inhibited by the inhibition of bacterial poison invasion into the host cell (Klasse and Sattentau 2002). Opsonization is basically the coating of a pathogens surface with antibodies for easier phagocytic activity. In this process, the engulfment of pathogens by phagocytic cells serves as a protective barrier against bacteria reproducing outside the host cell which are aimed at destroying the host (Vogt, Dowd et al. 2011). This can be achieved either by Fc receptors of phagocytic cells recognizing coated pathogens or by the adherence of antibodies to the pathogens surface for stimulation of proteins of the complement system (Hessell, Hangartner et al. 2007). Subsequently, this system uses these proteins to opsonize the pathogen by binding complement receptors on phagocytes. Moreover, phagocytic cells can be directed to the place of infection and terminal components of the system can destroy some microorganisms by creating pores in their membranes (Janeway and Travers, 2001).

Calnek (2001) and Davison and Nair (2004) have highlighted the vital role of antibody production against MDV glycoproteins, including gB, gE, and gI. However, it is the anti-gB neutralizing antibodies that aid in terminating viral invasion into the host cell (Schat and Markowski-Grimsrud, 2001) by nullification of the cell-free virus and activation of antibody-dependent cell-mediated cytotoxicity. Calnek (2001) reported that chicks can inherit passed down maternal antibodies from vaccinated or infected hens, and these antibodies assist in modifying MDV pathogenesis. Maternal antibodies can also be used to inhibit

viral replication, cancer transformation, death, and the ruthlessness of MD symptoms (Davison and Nair 2004; Witter and Lee 1984). Tohidi, idris et al. (2012) reported the significant association of the IFN-γ mutation at the -318 bp position with reduced SE counts through the induction of primary and maximum secondary antibody titres. This suggests the potential role of polymorphisms of cytokine genes, in improving resistance to disease.

2.6.5 Cell-mediated immunity

In 1988, Morimura showed that the absence of CD8+ T cells in a host cell produces a higher expression and titre concentration of MDV in CD4+ T cells (Morimura, Ohashi et al. 1998). Moreover, Omar and Schat (1996) proved that the cytotoxic activity of CD8+ T cells helps in reducing the titre concentration of MDV in cells expressing pp38, Meq, ICP4 and gB. Furthermore, they proved that the presence or the absence of CD4+ T cells and T cell receptor (TCR)-1+ cells does not necessarily help in eradicating MDV antigens. Hence, such findings highlight the importance of T cell-mediated immune responses against MD.

Cytokines have also been reported to mediate cell-mediated immune responses against MDV infection, particularly type 1 cytokines such as, IFN-γ, IL-2, IL-12 (Haq, Schat et al. 2013). The degree at which cytokines and chemokines are expressed against MDV is dependent on a few factors, such as the different phases of MDV pathogenesis, the genetic background of a bird, and the different cell and tissue populations. For instance, Xing and Schat (2000b) reported the enhanced expression of IFN-γ in MDV-stimulated spleen of chicken. In addition, Quere et al. (2005) also reported a high level of IFN-γ expression against MDV in resistant chicken lines which was not observed in the susceptible line. However, this was debated by Kaiser et al. (2003) who reported that the expression of IFN-γ was immunologically compatible in both resistant and vulnerable lines of chicken. This remains a controversial issue, highlighting the imperative need to analyse the immunological role of IFN-γ in genetic resistance to MD. Additional studies have analysed the expression of cytokines IL-1β and IL-8, and IL-6 and IL-18 in resistant and susceptible lines, respectively. Kaiser et al. (2003) reported a stimulated degree of expression of IL-1β and IL-8 in the splenocytes of resistant lines, and that of IL-6 and IL-18 in the splenocytes of vulnerable lines. These findings highlight the persisting expression of these particular cytokines against MD in their respective lines. Furthermore, Jarosinki et al. (2005) decided to infect chickens with a very virulent plus (v++) MDV strain or a virulent (vv) MD strain, and they monitored the degree of expression of cytokines, IL-1β, IL-6, iL-18 and IFN-γ in their brains (Jarosinski, Njaa et al. 2005). A directly proportional relationship was observed between the degrees of

cytokine expression with the virulence of MDV. A greater degree of cytokine expression (particularly IL-6 and IFN-γ) was observed in very virulent plus chickens compared to those infected with a less virulent strain (vv) of MDV (Haq, Schat et al. 2013). Hence, genetic background and different phases of MDV pathogenesis affect the level of expression of cytokine genes.

2.7 Candidate gene resistance cytokines

2.7.1 Interleukin-2

The interleukin-2 (IL-2) cytokine was first exposed around 30 years ago and it is generally produced by activated T lymphocytes found in the bone marrow. This cytokine was declared as a participant in growth promoting activity (Morgan, Ruscetti et al. 1976). IL-2 was one of the first cytokines to be successfully characterized at a molecular level. The IL-2 gene was cloned for the first time by Devos, Plaetinck et al. (1983), and its crystal form was first elucidated in 1992 by Bazan and McKay (1992). The ChIL-2 gene and the receptor of these genes have been successfully cloned and characterized in Muscovy ducks (Zhou, Wang et al. 2005) and (Teng, Zhou et al. 2006).

IL-2 is a soluble monomeric glycoprotein which weighs about 15kDa. The structure of this protein is globular and it consists of four α-helices folded in a similar manner to the configuration of the Type 1 cytokine family (Table 1). The 3D-structure of the Type I cytokines consists of 15 amino acid α-helices in characteristic arrangement. Only one disulfide bond is found between cysteines 58 and 105, and these are vital for cross-linking the second helix to the inter-helical region between the third and fourth helices and this is necessary for stability of the cytokine's structure. IL-2 is mapped on chromosome 4 and 3 of humans and mice, respectively (Gaffen and Liu 2004), whilst in chicken it is also mapped on chromosome 4 (Kaiser and Mariani 1999). This cytokine gene is a potential candidate in genotyping for disease resistance in the current study.

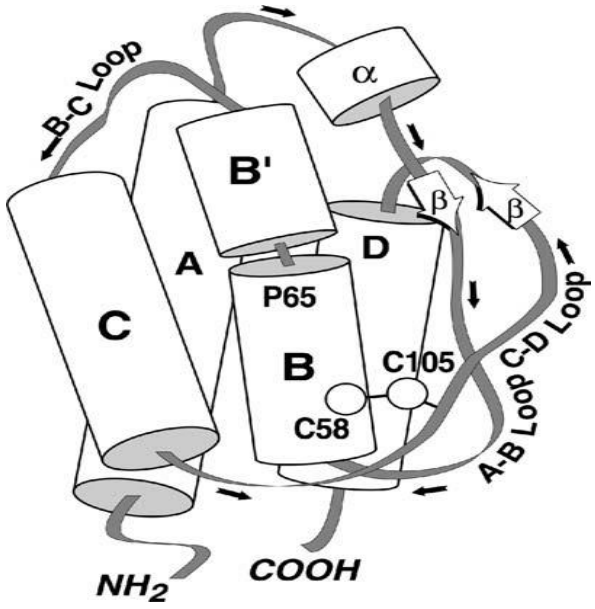

Figure 2.1: The IL-2 3D crystal structure in humans showing the position of the disulfide bond (Bazan and McKay 1992).

The chicken IL-2 (ChIL-2) gene has an exon-intron structure similar to the IL-2 gene of humans, thus is said to be homologous. However, the exon 2 and introns 2 and 3 of chickens are shorter. The exon 4 untranslated region of this gene possesses five ATTTA motif repeats. ChIL-2 is an individual-copy gene with no single nucleotide polymorphisms and/or promoter sequence polymorphisms identified. The chicken promoter region consists of regulating sequences such as, the 5' to 3' composite NF-AT/"AP-1" element, a CD28 response element, an AP-1 element, an NF-AT element, and the AP-1 part of an AP-1/octamer composite binding element. It is the polymorphism of the *Taq* I restriction enzyme that has enabled the mapping of the IL-2 gene on chromosome 4 (Kaiser and Mariani 1999).

The IL-2 cytokine has an imperative role in the immune system as it governs the three main processes of lymphocytes, including replication, maturation and differentiation of T- and B-lymphocytes (Gu, Teng et al. 2007) together with the duplication and maturation of natural killer cells (Choi and Lillehoj 2000). The ChIL-2 cytokine has also been used to enhance the effective response of vaccines to *Eimeria* parasites such as *Eimeria tenella* (*E. tenella*) and *E. acervulina* (Xu, Song et al. 2008) and infectious bursal disease virus (IBDV) by using the VP2 gene of IBDV as a DNA vaccine (Hulse and Romero 2004).

2.7.2 Immunological role of IL-2

The functional doings of IL-2 can be demonstrated on a variety of cell populations (Table 1), but T lymphocytes are the main cells answerable for IL-2 expression. The de novo production of IL-2 is the causing factor of the hastiest results of T cell activation through its antigen receptor. This is subsequently followed by the expression of the high affinity IL-2 receptor, thus enabling fast and selective expansion of T cell populations activated by antigen (Lenardo, Chan et al. 1999). IL-2 plays a vital role in triggering proliferation of CD4+ and CD8+ T cell types, through the pro-proliferative signals through proto-oncogenes such as c-myc and c-fos when combined with anti-killing signals through Bcl-2 family members (Miyazaki, Liu et al. 1995). Moreover, this cytokine promotes better survival capabilities of T cells by stimulating effects on cellular metabolism and glycolysis (Rathmell, Vander Heiden et al. 2000) and (Frauwirth and Thompson 2004).

Table 2.1: Showing a summary of cell types involved in IL-2 production (Gaffen and Liu 2004).

Cell type	Major functions of IL-2
CD+ T cells	▪ Promotes the enlargement of Ag-specific clones together in a proliferative and anti-aptotic manner. ▪ Promotes the synthesis of additional cytokines needed for differentiation to Th1 and Th2 subsets. ▪ Promotes the kill of activated T cells via Fas/FasL signaling ▪ Assists in the growth of CD4+CD25+ T regulatory cells.
CD8+ T cells	▪ Promotes the enlargement of Ag-specific clones. ▪ Boosts the emission of cytokines. ▪ Boosts cytolytic function. ▪ Promotes proliferation of memory CD8+ cells.
B cells	▪ Augments Ab excretion. ▪ Primes IgJ chain transcription and synthesis. ▪ Augments proliferation.
NK cells	▪ Induces proliferation. ▪ Boosts the production of cytokines. ▪ Augments cytolytic function.

In contradiction, IL-2 has demonstrated a purpose of down-regulating immune responses in knockout mice so as to prohibit autoimmunity. These effects cause the formation of a negative feedback loop through a couple of mechanisms. One of these include the synthesis of impermanent IL-2, which is problematic because in the occurrence of persistently produced antigens the apoptosis of T cells is triggered due to the impermanent production of IL-2 to the surrounding environments. Moreover, IL-2 activates an early kill mechanism through promoted expression of FasL on activated T cells (Refaeli, Van Parijs et al. 1998), and because T cells also express Fas/CD95; programmed apoptosis of T-lymphocytes is triggered. There are several of studies that propose the role of IL-2 in thymic progress which in turn inhibits autoimmunity by promoting the development of CD4+ an CD25+ T regulatory cells (Malek, Porter et al. 2000; Malek, Yu et al. 2002).

IL-2 and IL-15 act as a growth factor for NK cells, and both these interleukins are known to share the same receptor in signaling (Imamichi, Sereti et al. 2008). Although they use the same receptor they

demonstrate different effects in vivo (Ma 2000). IL-2 moreover induces the expression of a variety of cytokines and chemokines or their receptors. The synthesis of NK-derived cytokines such as, TNF-α, IFN-γ and GMCSF is stimulated by IL-2. A synergistic connection between IL-2 and IL-12 has been reported, and both these cytokines promote NK cytotoxic activity when in synergy (Khatri, Fehniger et al. 1998). The IL-2 cytokine is also immunologically involved in B cells by promoting antibody release. The synergistic effect of IL-2 and IL-5 combination promotes the expression of heavy and light chain genes in immunoglobulin (Ig)-M expressing B cells, and also promotes the de novo production of the IgJ chain gene (Blackman, Tigges et al. 1986). This activity is needed for oligomerization of the IgM pentamer, and this appears as well in the controlled stage of B cell activation (Koshland 1985). IL-2 functions similarly in B cells as in T cells by inducing the expression of IL-2Rα which in turn induces their responsiveness to IL-2 (Gaffen, Wang et al. 1996).

2.7.3 Cell and tissue populations that express IL-2

There are a number of cells and tissues that express IL-2 (Table 1). However, CD4+ T cells are the main expressers. An estimated 60% of activated CD4+ T cells trigger the expression of IL-2 after vague stimulation by treatment using phorbol 12-myristate 13 acetate (PMA) and a calcium ionophore or antibodies that conjoin CD3 and CD28. On the other hand, a great amount if not all T cells synthesize IL-2 straight after antigen stimulation. It is only the T helper (Th)-1 cell subsets that manufacture IL-2 in great quantities subsequently to Th cell differentiation (HO, Kim et al. 1999). IL-2 in CD4+ T cells activates differentiation to Th1 and Th2 subsets thus, promoting the death of activated T lymphocytes, and also assists in the development CD4+ CD25+ Tregs (Burchill, Yang et al. 2007). CD8+ T cells are involved in generating moderate amounts of IL-2 following the stimulation of their T cell receptors. The IL-2 of CD8+ cells mediates cytotoxic activity and stimulates memory CD8+ cell proliferation (Gaffen and Liu 2004). IL-2 essentially functions in modulating the expansion of Treg in the thymus, and in the absence of it, the IL-15 cytokine is capable of modulating Treg proliferation. The IL-7 γc-dependent cytokine also aids in this modulation. However, IL-2-dependent signaling causes the inactivation of IL-7Rα and IL-15Rα chains. Hence, the maturely developed Tregs primarily rely on IL-2 for survival and growth (Burchill, Yang et al. 2007). IL-2 is also manufactured by antigen presenting cells (APCs) in small amounts. For instance, B cell lines have demonstrated minor synthesis of IL-2 (Gaffen, Wang et al. 1996). Furthermore, IL-2 is produced in temporal scales by dendritic cells (DC) after microbe invasion, and because of this act, IL-2 was hypothesized as a contributory factor in inducing T cell activation. This can be reinforced by a finding that showed that DCs from IL-$2^{-/-}$ mice are incapable of inducing T cell proliferation, whilst macrophages seem not to synthesize IL-2 upon bacterial activation (Granucci,

Andrews et al. 2002). This shows that not every approach of T cell activation is dependent of IL-2 from APCs.

2.7.4 Pathways involved in IL-2 synthesis

There are two pathways that are used to activate the making of IL-2 from T cells; these include the T cell antigen receptor (TCR)/CD3 signals and the CD28 signals. TCR signaling also known as 'signal 1' achieves this by activating the phospholipase C (PLc) γ-dependent pathway which subsequently triggers the following transcription factors: the nuclear factor of activated T cells (NFAT), NF-κB, and AP-1. However, this signaling is incapable of producing the maximal amount of IL-2. Therefore, signaling through the CD28 costimulatory molecules also termed as 'signal 2' is then activated for the maximal release of IL-2 (Isakov and Altman 2002). The TCR/CD3 signaling involves the recognition of the MHC antigen complex on APCs. However, the disadvantage is that the binding affinity of this complex is quite low, but in the presence of several accessory molecules the interactive relationship between the T cell and APC is enhanced (Huppa and Davis 2003). Moreover, costimulatory molecules (CD28) assist in the synthesis of significant levels of IL-2 by binding the B7-1 and B7-2 of APCs (Kane, Lin et al. 2002).

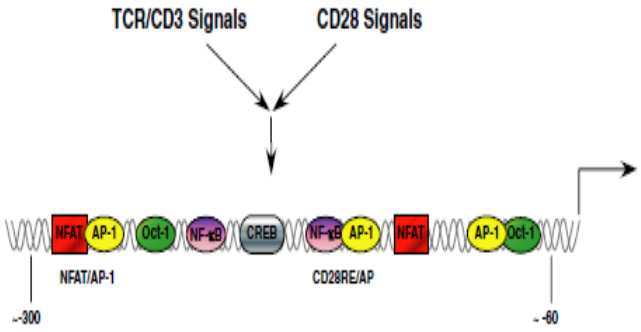

Figure 2.2: Diagram showing the 5' upstream region of the IL-2 gene and the two pathways involved in the production of IL-2 (Gaffen and Liu 2004).

There are several other pharmacological enhancers and suppressors that have been revealed that induce TCR signaling. These include the use PMA and Ionomycin to non-specifically induce T cells for significant levels of IL-2 production. The PMA enhancer initiates the TCR/CD3 complex signaling for IL-2 synthesis and this enhancer is an analog of a messenger molecule known as diacylglycerol

manufactured by PLcγ. Another enhancer involved in this pathway is a calcium ionophore called ionomycin which aids in the successful intracellular transportation of calcium (Ca^{++}) ions (Gaffen and Liu 2004).

Drugs such as cyclosporine A (CsA), Rapamycin and FK506, and PDTC and SN50 are involved in the TCR/CD3 signaling pathway for inhibition of IL-2 production. The CsA drug is a very powerful cyclic oligopeptide that acts as an immunosuppressant in the signaling pathway by suppressing calcineurin activity needed for the transportation of NFAT into the nucleus for IL-2 production. The Rapamycin and FK506 are also involved in this suppressing activity of calcineurin using a different mechanism (Brown and Schreiber 1996). The PDTC and SN50 inhibitors of the NF-κB pathway aid in suppressing IL-2 synthesis, and these effects can be used for clinical implications (Martínez-Martínez, del Arco et al. 1997; Lin, Yao et al. 1995).

2.7.5 The IL-2 Receptor (IL-2R)

IL-2 is mainly synthesized by T lymphocytes and this cytokine confers its immunological role by binding the high affinity IL-2R. This receptor is made up of three subunits which possess different levels of affinity (high, intermediate and low) for binding to their respective ligands. The subunits are as follows: the α-chain with the IL-2Rα/CD25, the β-chain with the IL-2Rβ/CD122 and the γ-chain with the IL-2Rγc/CD132. The latter chain is also known as the common cytokine receptor. All of these subunits aid in IL-2 binding, but only the IL-2Rβ (p75) and the IL-2Rγc (p65) are vital for effective signal transduction (Bazan 1990; Ozaki and Leonard 2002). The IL-2Rα has a low affinity receptor which is homologous to the IL-15Rα complex. The IL-2Rα is proficient of inducing the affinity of IL-2R for ligand by almost one hundred fold.

The “IL-2 family” has been identified as the great amount of cytokine receptors that also use the subunits of the IL-2R. The IL-2Rα is mainly used by IL-2R, the IL-2Rβ chain is a vital part of the trimeric IL-15R, and the IL-2Rγ subunits are involved in IL-4, IL-7, IL-9, IL-15 and IL-21 receptor complexes (Ozaki and Leonard 2002). It is essential to know that the ability of IL-15R to utilize both IL-2Rβ and IL-2Rγc subunits causes IL-15 to participate in vastly comparable or the same signaling pathways in target cells (Giri, Ahdieh et al. 1994).

2.7.6 Interleukin-12 (IL-12)

IL-12 is a heterodimeric protein made up of two conjoined disulfide subunits, the p35 and the p40. The p35 is an α-subunit whilst the p40 is a β-subunit (Kobayashi, Fitz et al. 1989), and both these subunits are encoded by unalike genes (Stern, Podlaski et al. 1990). These genes are located on chromosomes of both human and mouse genomes. For instance, IL-12p35 and IL-p40 are found on chromosomes 3p12-3q13.2 and 5q31-33 of the human genome (Sieburth, Jabs et al. 1992), and chromosomes 3 and 11 of the mouse genome, respectively (Trinchieri 1994; Noben-Trauth**,** Schweitzer et al**.** 1996). There is no sequence homology that has been reported between these two chain subunits, but individually the p35 subunits has demonstrated sequence homology with other cytokines, including IL-6, the granulocyte-colony-stimulating factor (CSF), and the chicken myelomonocytic growth factor (Merberg, Wolf et al. 1992). However, the p40 subunits have no sequence homology but possesses similar structure to the IL-6 receptor and ciliary neutrophic factor receptor extracellular domains (Gubler, Chua et al. 1991). Both these subunits can form dimers with IL-12 moreover, the p40 subunit can create dimers with its counter parts. For example, IL-12$(p40)_2$ or IL-12p80 (Ling, Gately et al. 1995). Although unalike, the genes encoding for the α- and β-subunits should be expressed within the same cell so as to motivate the production of their actively conjoined heterodimer, called p70. There is a possibility for this heterodimeric complex (p70) not to form due to inaccessibility of the α-chain, which will cause two β-subunits to mingle forming the p80 peptide. Unfortunately, the p80 peptide is also termed the IL-12 antagonist since it contends for the hetorodimeric linkage between the IL-12 R and its counter cytokine (IL-12). Thus, inhibiting Th1 immune responses of this heterodimeric complex (Ling, Gately et al. 1995) and/or permitting antagonistic cell-mediated immune responses (Hölscher, Atkinson et al. 2001) and (Piccotti, Li et al. 1998).

2.7.7 Polymorphisms of IL-2

IL-2 aids in the proliferation and differentiation of T, B and natural killer (NK) cells (Janeway, Travers et al. 1997) and this gene has been suspected to assist in reducing the burden of *Salmonella* in different tissue organs. Kramer investigated the association of twelve candidate gene polymorphisms and their response with SE in five different meat types, and this study confirmed the vital role of IL-2 of reducing SE counts in tissue organs (Kramer, Malek et al. 2003). Kaufmann, Medzhitov et al. (2003) discussed the role of IL-2 in reducing SE counts, whereby he highlights the necessity of the IL-2 dependent pathway for the activation of NK cells. In this process, $CD8^+$ $CD44^{high}$ cells express an adaptor molecule called DAP_{12}

which is usually expressed by activated NK cells (Dhanji and Teh 2003), and in turn assist in the prohibition of SE count domination in the caecum. Many studies have reported different functions of the IL-2 gene in immunity and the significance of the genes genetic polymorphisms in susceptibility and resistance to disease. In 2016, Maddah reported the significant association of IL-2 -330 GG genotype ($p<0.01$) and IL-3 -330 and +166 GT haplotype with individuals' susceptibility to Juvenile Idiopathic Arthritis (Maddah, Harsini et al. 2016). Moreover, gene polymorphisms of IL-2 are known to potentially affect individual susceptibility to Juvenile Systemic Lupus Erthematousus (JSLE), and its potential ability to take on the role of possible genetic markers for vulnerability to JSLE (Harsini, Ziaee et al. 2016). In the presence of the IL-2 -330 GG genotype, polymorphisms of this locus can increase the risk of Basal Cell-Carcinoma (BCC) and influence the clinical course of BCC in Polish population (Sobjanek, Zablotna et al. 2016). The IL-2 gene polymorphism (rs6534349) has been potentially labeled as a biomarker that can be utilized for estimating the risk of asthma and Mycoplasma pneumonia infection in children (Wang, Jin et al. 2015), and as a potential biomarker for the incidence of Graves' disease (Liang, Du et al. 2015). Moreover, the rs2104286 polymorphism of the IL-2RA is associated with rheumatoid arthritis (RA) persistence, and it is the only genetic variant that has been discovered to be linked with both joint destruction and RA-persistence so far. This highlights the importance of IL-2RA for RA. IL-2 is a cytokine bound by the IL-2 receptor (IL-2R). This cytokine is usually expressed in high quantities in both Salmonella infected and Salmonella free chickens by cells that possess its receptor. These cells include, $CD8_{\alpha\alpha}^{+}$ γδ T cell (Pieper, Methner et al. 2011). The IL-2R is a multipurpose receptor that aids in cell activation for the synthesis and high excretion of cytokines, and the proliferation and extended endurance of lymphocytes. It is believed that the latter functions of the receptor towards memory cells is a result of the high expression of the $CD8_{\alpha}$ chain and $IL\text{-}2R_{\alpha}$ transcription. The IL-2R additionally elucidates the final apoptotic activity of T cells and memory development (Schluns and Lefrançois 2003, Rocha and Tanchot 2004).

2.7.8 Adjuvant effect of IL-2

Li, Tang et al. (2015) exploited the adjuvant effect of chicken IL-2 (chIL-2) against *Eimeria mitis* (*E. mitis*) infection by developing an *E. mitis* line that express the chIL-2 cytokine, and using *E. mitis* as a vaccine strain against its own infection. Findings of this study revealed that *E. mitis* chIL-2 was able to induce more effective immune responses against infection compared to the wild-type parasite. This proves that *E. mitis* chIL-2 can be used as a potential vaccine strain against coccidiosis and as a harmless and operative adjuvant cytokine. The *E. mitis* line helped to reduce the *E. mitis* replica cycle which in turn

assisted in removing the quantity of parasites harbored in cells. This was achieved by the use of an actin gene promoter that governed the continuous expression and excretion of the E. mitis chIL-2 expressing lines (Yin, Liu et al. 2011). Induced immunogenicity assists vaccinated chickens to develop stable and effective immunity rapidly, thwarting vaccination repercussions by avoiding re-introduction to infections. Therefore, *E. mitis* anticoccidial vaccine strains are promising for harmless use in chickens (Li, Tang et al. 2015). In 2014, Kim developed a recombinant NDV vectoral experimental norovirus (NoV) vaccine to overcome the inability to grow human NoV in cell culture for the development of live-attenuated vaccines (Kim, Chen et al. 2014). Findings showed that the Rndv vectors induce Th1 response by promoting IgH2a subclass Ab, thus splenocytes of chicken demonstrated high levels of IFN-γ, IL-2 and TNF-α. (Maughan, Dougherty et al. 2013) reported significant induction of both IL-2 and IFN-γ cytokine transcription against H7 AIV isolate infection.

2.7.9 Immune system development and IL-2 expression

Full development of the immune system usually exists between day 2 and 4 post hatching (Lowenthal, Connick et al. 1994). For instance, an *in vitro* study proved that splenic T cells of day-old chickens are unable to synthesise IL-2 compared to 7-day old post hatch chickens. Crhanova moreover demonstrated induced expressions of IL-8 after 3 days' post hatch in the caecum (Crhanova, Hradecka et al. 2011). Therefore, it is important to note that time is a confounding factor because some tissue organs show significant associations with SE counts at different time intervals. Moreover, the age of a chicken together with the infection dose are also confounding factors because they both can affect how a chicken responds to an infection. IL-2 also aids in up-regulating particular activities of heterophils. Kogut, Rothwell et al. (2002) reported the disabled function of heterophils at a day old post hatch, but increased phagocytic and bactericidal activity from day 7 to 14 post hatch ($P<0.01$). Heterophils and other immune cells are famous for generating low levels or no cytokines at all during the first seven days' post hatch, and this increases the vulnerability of a chicken to infection (Oppenheim and Shevach 1991). Zhou, Buitenhuis et al. (2001) investigated the ability of chicken heterophils treated with recombinant chicken IL-2 (rChIL-2) to phagocytize non-opsonized SE compared to the effect of mock transfected cell supernatants. In this study, day old chicken heterophils were unable to demonstrate neither phagocytic nor bactericidal activity compared to two-week old post hatch chickens. Phagocytic activity depended on the amount of heterophils that phagocytized SE and the amount of SE engulfed by an individual heterophil. Moreover, the ability of IL-2 to promote phagocytic activity was shown by the presence of specific anti-ChIL-2 monoclonal antibodies to abrogate the phagocytic activity of cells (Zhou, Buitenhuis et al. 2001).

Inconsistencies of results with different investigative studies can be a result of confounding factors such as, environmental effects, populations, evolutionary processes in the immune system, challenge with different strains of Salmonella, and the genetic upbringing of the chickens. These inconsistencies prove that when using markers in a selection program it should be on the basis of association analysis in the population.

2.7.10 Regulation of IL-12

There are various cell populations that remain accountable for synthesizing IL-12, these include macrophages, monocytes, dendritic cells, neutrophils, and B cells (Watford, Moriguchi et al. 2003). All these cell types are major producers of IL-12 except for B cells that produce minimal amounts, and the major actions of IL-12 occur within the NK- and T-cells (figure 3). IL-2 has previously demonstrated powerful combinatorial immunology with cytokines IL-2 (Gaffen and Liu 2004) and IL-18, acting on macrophages and dendritic cells to kindle the synthesis of IFN-γ, also in APCs.

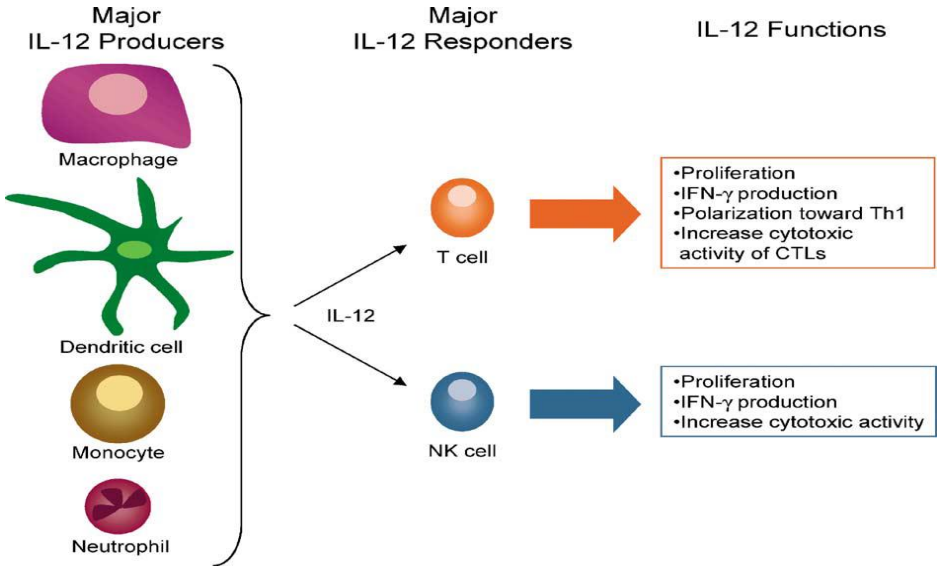

Figure 2.3: Showing the producers, major responders and the immunological roles of IL-12 (Watford, Moriguchi et al. 2003).

The expression of the p35 peptide differs from that of p40 in that it occurs in a ubiquitous and constitutive manner in low levels, whilst that of p40 only occurs in phagocytic cells that manufacture IL-12p70. The p40 gene is excessively expressed by microbial products and is transcriptionally facilitated. Several transcription factors, including NF-κB, IRF-1, C-Re1, interferon consensus binding protein (ICSBP), and Ets family members attach themselves to the p40 promoter region of human and mice, respectively (Ma, Chow et al. 1996; Plevy, Gemberling et al. 1997). The p40 gene is made up of an Ets region (from -211 to 207) (Ma, Neurath et al. 1997) and an NF-κB half-region (from -117 to -107) (Murphy, Cleveland et al. 1995) which are cis-regulatory elements. Mutations or any deleterious activity that may occur in these regions can interfere with the p40 promoter activity (Gri, Savio et al. 1998). The Ets-region aids in recruitment of a large complex made up of different transcription factors, including C-Re1, Ets-2, IRF-1, and Ets-2 related protein. The ICSBP factor also binds to the Ets region, and mice consisting of insufficient levels of IRF-1 (Salkowski, Kopydlowski et al. 1999) or ICSBP (Giese, Gabriele et al. 1997) confer impaired production of IL-2. The NF-κB region interacts with both p50/p65 and p50/c-Re1 heterodimers.

As mentioned that p35 is ubiquitously and highly expressed than p40 which is restricted to phagocytic cells for its expression, the IL-12p35 complex can also be regulated transcriptionally and translationally. Different transcriptional sites aid in manufacturing p35 mRNA transcripts with different translational efficiencies (Babik, Adams et al. 1999). In the existence of an inhibitory ATG within the 5'-untranslated region; only minimal amounts or no protein at all can be synthesized but in the presence of induced lipopolysaccharide (LPS); transcription is activated which further promotes translation for protein production (Babik, Adams et al. 1999).

The high production of IL-12p40 and IL-12p70 is controlled by a number of pathogenic organisms, such as bacteria (Gram negative and Gram positive), parasites, viruses and fungi. The production of IL-12 can either occur in an independent or a dependent fashion. In independent production, microbial products such as, lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan and bacterial (CpG) DNA promote the T cell-independent synthesis of IL-12, via the recognition of innate cells by TLRs. Whilst in dependent synthesis, an APC CD40 molecule is recruited together with its cognate receptor (CD40L) on T cells (Jacobson, Szabo et al. 1995; Cella, Scheidegger et al. 1996). IFN-γ serves in modulating the production of IL-12, and IL-12 is responsible for IFN-γ enhancement through participation in a positive feedback loop, which in turn highly activates monocytes and PMN for a greater synthesis of IL-12 (Ma, Chow et al. 1996; Kubin, Chow et al. 1994). The synthesis of IL-12 can be disturbed in the presence of IL-10, IL-11, IL-13 and other sort of cytokines (such as type 1 IFNs). There is a controversial issue on the activity of IL-4 in suppressing or inducing IL-12 production. IL-4 seems to have a dual function of either

inhibiting IL-12 production (Major, Fletcher et al. 2002) or promoting IL-12 generation (D'Andrea, Ma et al. 1995; Takenaka, Maruo et al. 1997; Hochrein, O'Keeffe et al. 2000). Furthermore, other antagonists of IL-12 synthesis include, G-protein coupled receptors (GPCR) such as the receptors for monocyte chemo-attractant protein 1 (MCP-1), prostaglandin E2, histamine and FcR crosslinking, except for a GPCR called CCR5 which acts as an inducer for this synthesis (Aliberti, 2002). In the presence of cholera toxin (CTX) and measles, the production of IL-12 is suppressed.

2.7.11 IL-12 Receptor (R)

The IL-12R is made up of two β-subunits (β1 and β2), thus are designated as IL-12Rβ1 and IL-12Rβ2. The IL-12R depends on activated NK- and t-cells for its expression (Desai, Quinn et al. 1992). The IL-12Rβ1 and IL-12Rβ2 have estimated molecular weights of 100kDa and 130kDa, respectively. Both these subunits individually bind in low affinity to IL-12 in humans permitting high affinity binding sites of IL-12 (Wilkinson, Carvajal et al. 1997). The IL-12R subunits in both humans and mice are known to display sequence homology, however this is not the case when analyzing them as individuals to their counterparts (Bacon, 1995**)**. For instance, the IL-12R subunits in mice can confer both low- and high-affinity binding with mouse IL-12, and the human IL-12Rβ2 confers low-affinity binding with IL-12 whilst the mouse counterpart subunit confers extremely low-affinity binding even hard to quantitate. Hence, both these receptor subunits show dissimilar binding affinities to IL-12. It has been recommended that IL-2 needs the interaction of IL-12Rβ1 and p40, and that of IL-12Rβ2 and p35 in order to establish its functional role (Presky, Yang et al. 1996). It has been reported that IL-12Rβ2 aids as a signal transducer of the high-affinity receptor complex (Presky, Yang et al. 1996) (Zou, Yamamoto et al. 1995) which means that the mouse IL-12Rβ2 aids in signal transduction whilst the IL-12Rβ1 delivers the utmost binding energy. However, in humans both the IL-12R subunits contribute to the binding energy whilst the IL-12Rβ2 acts as the initial signal transducer. The mingling that occurs between IL-12 and its high-affinity receptor stimulates the transphosphorylation of the Janus kinases (Jak2 and TYK2) (Bacon, McVicar et al. 1995), together with STAT1, STAT3, STAT4 and STAT5. However, it is the phosphorylation of STAT4 that regulates the cellular effects of IL-12 (Bacon, McVicar et al. 1995). When phosphorylated, the IL-12β2 receptor chain provides a binding site for STAT4, which is then phosphorylated. STAT4 homodimers are then transported to the nucleus for exposure to the IFN-γ promoter region STAT binding sites, and thereafter the induced transcription of the IFN-γ gene (Figure 4a) (Hölscher 2003) (Watford et al., 2003). The IL-12Rβ2 cytoplasmic tail provides particular tyrosine for the recruitment of STAT4. For instance, in human's tyrosine 800 is needed for STAT4 recruitment in response to IL-12 whilst mice need tyrosine

757, 804 and 811 for the phosphorylation of STAT 4 (Figure 4b, Reviewed by Watford et al.2003). It is believed that the IL-12Rβ2 affinity binding may be restricted to just Th1 cells and the expression of this receptor complex for IL-12 responsiveness in Th1 cells (Rogge, Barberis-Maino et al. 1997). Subsequently, differentiation then occurs and the Th2 cells express IL-12Rβ1 but not IL-12Rβ2 (Rogge, Barberis-Maino et al. 1997). Moreover, the expression of IL-12Rβ2 is chiefly controlled by the presence of IL-10, TGF-β cytokines and NO (Diefenbach, Schindler et al. 1999), which in turn may regulate IL-12 responsiveness and Th1 development.

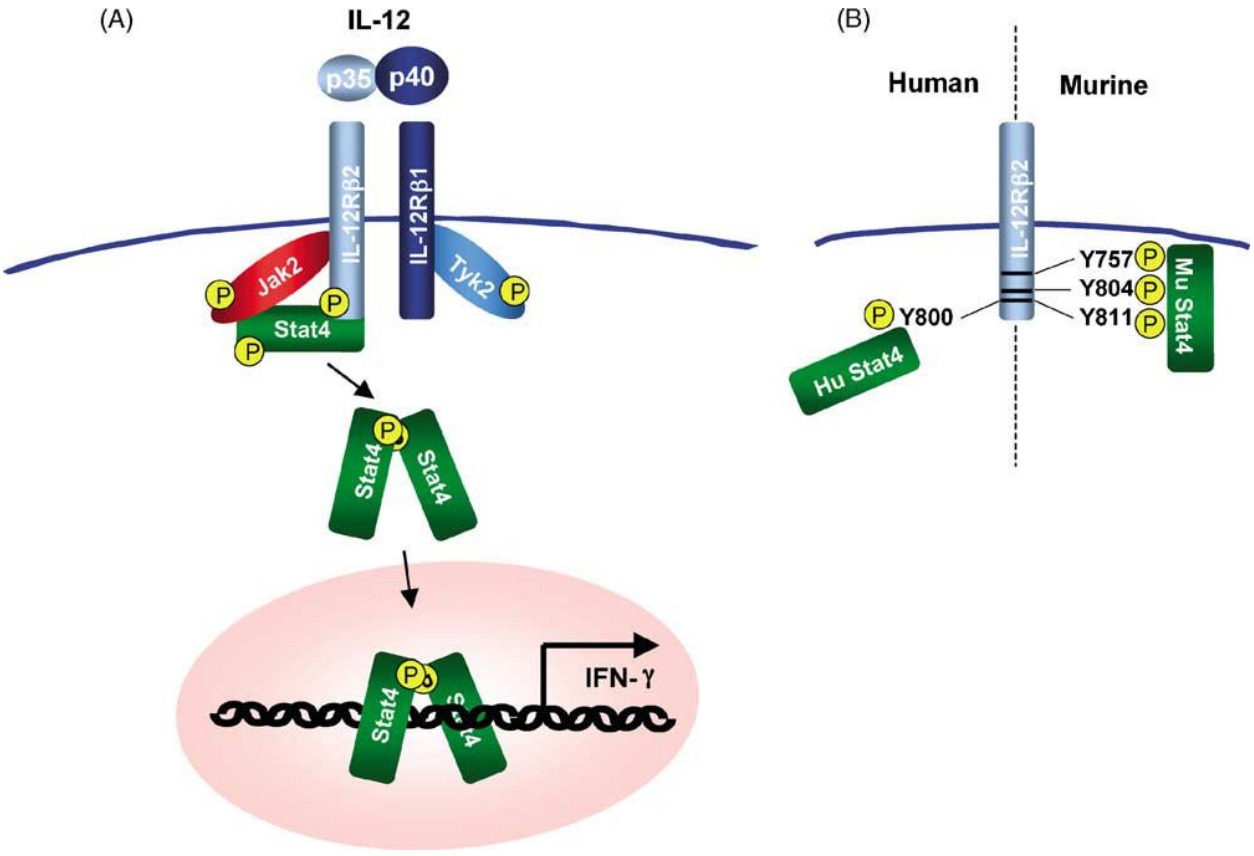

Figure 2.4: Diagram showing the IL-12 signal transduction, (A) the bond between IL-12Rβ2 with IL-12Rp35 and IL-12Rβ1 with IL-12Rp40 for the functional role of IL-12, and the interaction of IL-12 with its receptor for the phosphorylation of Janus kinases. Highlighting the phosphorylation of STAT4 as the main regulator of IL-12 cellular effects. The IL-12Rβ2 cytoplasmic tail tyrosine needed for STAT4 recruitment and phosphorylation in humans (tyrosine 800) and mice (tyrosine 757, 804 and 811) (Watford, Moriguchi et al. 2003).

2.7.12 The effect of IL-12 in intracellular infection

IL-12 is manufactured by APCs in the innate immune system so as to regulate cell-mediated immune responses which in turn form the first line of defense for the host contrary to invading intracellular pathogens (Trinchieri 1995). It is the recognition of PAMPs by Toll-like receptors (TLRs) that aids in activating APCs for IL-2 synthesis (Reiling, Hölscher et al. 2002; Medzhitov and Janeway 1997). The imperative role of IL-12 in regulating cell-mediated immune responses can be observed with the genetic scarcities of IL-12 or IL-12R components in human and mice. Suppressing mutations that are found within these genes can disturb cellular immunity, thus promoting the host's vulnerability to intracellular infections (Magram, Sfarra et al. 1996; de Jong, Altare et al. 1998; Altare, Durandy et al. 1998; Wu, Wang et al. 2000). The host relies on the significant activation of macrophages by the IFN-γ dependent mechanisms to establish cell-mediated immune responses against infections (Raupach and Kaufmann 2001). IFN-γ is a pleiotropic cytokine responsible for inducing the antimicrobial activity of macrophages which is then followed by the destruction of intracellular pathogens by iNOS reactive nitrogen intermediate (Hölscher, Köhler et al. 1998). The activation of NK- and T-cells induces the synthesis of IFN-γ by IL-12 acting as the growth factor for NK cells, thus promoting the production of cytotoxic CD8+ T cells (Trinchieri 1994). In vivo, IL-12 is highly involved in three stages of innate resistance and the adaptive immune response against infection. NK cells and T cells aid in the early synthesis of IFN-γ straight after infection, and this is necessary for macrophage stimulation and inflammation. Both IL-12 and IL-12 induced IFN-γ promote Th1 cell differentiation by stimulating Th1 cells to synthesize excessive amounts of IFN-γ. Moreover, IL-12 aids in the maximal synthesis of IFN-γ and proliferation of antigen-specific differentiated Th1 cells in response to APCs. Consequently, IL-12 serves as a linking bridge between early non-specific innate resistance and the antigen specific-adaptive immune response to invading pathogens.

2.7.13 Insufficiencies of IL-12

IL-12-KO mice are vulnerable to several intracellular infections, such as those caused by *Leishmania major* (*L. major*), *L. donovani* (Satoskar, Rodig et al. 2000), *Toxoplasma gondii* (Ely, Kasper et al. 1999), *Trypanosoma cruzi* (Müller, Köhler et al. 2001), *Cryptococcus neoformans* (Kawakami, Koguchi et al. 2000), *mycobacteria* (Cooper, Magram et al. 1997), and *Listeria monocytogenes* (Brombacher, Dorfmüller et al. 1999). This is evident by the disturbed activity of NK, CD4+ and CD8+ T cells in various studies. Surprisingly, in the scarcity of IL-12 IL-12-KO mice preserve their ability to produce minute amounts of IFN-γ as Th1 immune response. This proves the functional activity of an IL-12

independent pathway of cell-mediated immune responses and a residual resistance in these mice. However, this was not the case for RAG-IL-12-KO mice lacking T cells. Owing to privation of T cells, the RAG/IL-12-KO mice portrayed an induced susceptibility status of infection by a 10-fold increase compared to IL-12-KO mice (Müller, Köhler et al. 2001). IL-12-KO mice are also resistant to viral infections such as mouse hepatitis virus (Schijns, Haagmans et al. 1998), systemic lymphatic choriomeningitis virus (Oxenius, Karrer et al. 1999), and pulmonary adenovirus (Xing, Zganiacz et al. 2000). In addition to the usual role of IL-12-KO mice in modulating cell-mediated immune responses and induction of Th1 differentiation, these mice may also take part in the assistance of memory immune responses with re-introduction of an infection (Stobie, Gurunathan et al. 2000). For instance, re-introduction of IL-12-KO mice that survived the first infection of L. monocytogenes to the same pathogen conferred resistance to the infection as well as the wild type mice did (Brombacher, Dorfmüller et al. 1999).

2.7.14 Insufficiencies of IL-12R

In the shortcoming of the IL-12R, both humans and mice portray weak cell-mediated immune responses. Hence, increased susceptibility to opportunistic infections is observed (de Jong, Altare et al. 1998; Wu, Wang et al. 2000). However, even in undersupplied amounts of IL-12R the host still produces minimal amounts of IFN-γ due to the activation of the IL-2 independent pathway that regulates consistent cell-mediated immune responses (Verhagen, de Boer et al. 2000). It would make sense to believe that mice lacking the p35 and p40 subunits would portray the same or similar phenotypes if both the subunits of IL-12 were to be exceptionally used by IL-12. This is evident with IL-12p35- and IL-12p40—KO mice that both demonstrated susceptibility to *L. major* infection, whilst the wild type mice successfully repaired the injuries resulting from the infection (Sacks and Noben-Trauth 2002). In Table 2.2, different responses to the infection by IL-12p35- and IL-12p40-KO mice are shown. The IL-12p40-KO mice demonstrated vulnerability to infections caused by mycobacteria (Hölscher, Atkinson et al. 2001) (Cooper, Kipnis et al. 2002), and the highest susceptibility levels to infections caused by *C. neoformans* (Decken, Köhler et al. 1998), *Salmonella enteritidis* (*S. enteritidis*) (Lehmann, Bellmann et al. 2001), and *Francisella tularensis* (*F. tularensis*) (Elkins, Cooper et al. 2002) due to reduced IFN-γ synthesis. A great number of mice fatalities and infection loads in the tissue organs indicated the vulnerability of mice to infection. In contrast, the IL-12p35-KO mice managed to facilitate cell-mediated immune responses even in the absence of IL-12, proving that the shortage of the p40 subunit displays a further immunodeficiency phenotype than the p35 deficit. Moreover, p40 may work independently of its heterodimeric complex

with IL-12 for protective function. Shockingly, IL-12p35-KO mice can still persistently synthesize adequate amounts of IL-12p80 (antagonist of IL-12) and IL-12p40 even in the absence of IL-12, which suggests that IL-12p80 or IL-12p40 monomer, alone or their association with a different polypeptide of IL-12p35 might function in pathogen eradication. Furthermore, the addition of exogenous IL-12p80 in IL-12p40-KO mice following a mycobacterial infection restores cell-mediated immune responses and resistance to IL-12p40-KO mice (Hölscher, Atkinson et al. 2001). The antagonistic property of the IL-12p40 homodimer may be the reason behind this activity, if not an unknown partner of p40 (Piccotti, Li et al. 1998).

Table 2.2: Showing a comparison between IL-12p35- and IL-12p40-KO mice to wild type mice based on relative resistance (pathogen reduction or survival) and IFN-γ manifestation in tissue organs or serum or after antigen-specific re-stimulation of T cells derived from infected animals. The pathogen burden or persistence is shown as (comparable =, reduced <, or highly reduced <<) and IFN-γ expression is also shown in the same manner (Hölscher 2003).

Pathogen	**IL-12p35-KO Res.**	**IL-12p35-KO IFN-y**	**IL-12p40-KO Res.**	**IL-12p40-KO IFN-y**
Franciscella tularensis	<		< <	
Mycobacterium tuberculosis	<	<	< <	< <
Mycobacterium bovis BCG	=	=	< <	< <
Salmonella enteritidis	<	<	< <	< <
Leishmania major	< <	< <	< <	< <
Trypanosoma cruzi (unpublished)	<	<	<	<
Cryptococcus neoformans	<		< <	

2.7.15 Role of costimulatory cytokines in IFN-γ synthesis

Luo, Chen et al. (2003) analyzed the role of Th1 and Th2 cytokines in BCG-induced IFN-γ production. The study analyzed the effects of different costimulatory cytokines, including IL-2, IL-6, IL-10, IL-12, IL-18, GMCSF and IFN-α and TNF-α. The effect of these cytokines for IFN-γ synthesis was analyzed as individuals, as an individual combination to BCG (e.g. IL-2+BCG combined), as a multiple combination to BCG (e.g. BCG+IL-2+IL-12), and as a multiple rational combination without BCG (IL-2+IL-12+IL-18+IFN).

Upon analysis of the cytokines; IFN-γ proved to be a late responsive cytokine to BCG in both the splenocytes of mouse and peripheral blood mononuclear cells (PBMCs) of human systems. This is because IFN-γ solely relies on the endogenous production of Th1 and Th2 cytokines after BCG stimulation before it can be synthesized. Figure 2.5a and 2.5b show IFN-γ being produced at a 2-day incubation time after an earlier production of IL-2, IL-6, IL-10, IL-12, IFN-γ, and TNF-α and GMCSF cytokines in the splenocytes and PBMCs respectively, of non-BCG immunized hosts. Hence, IFN-γ is termed the late responsive cytokine.

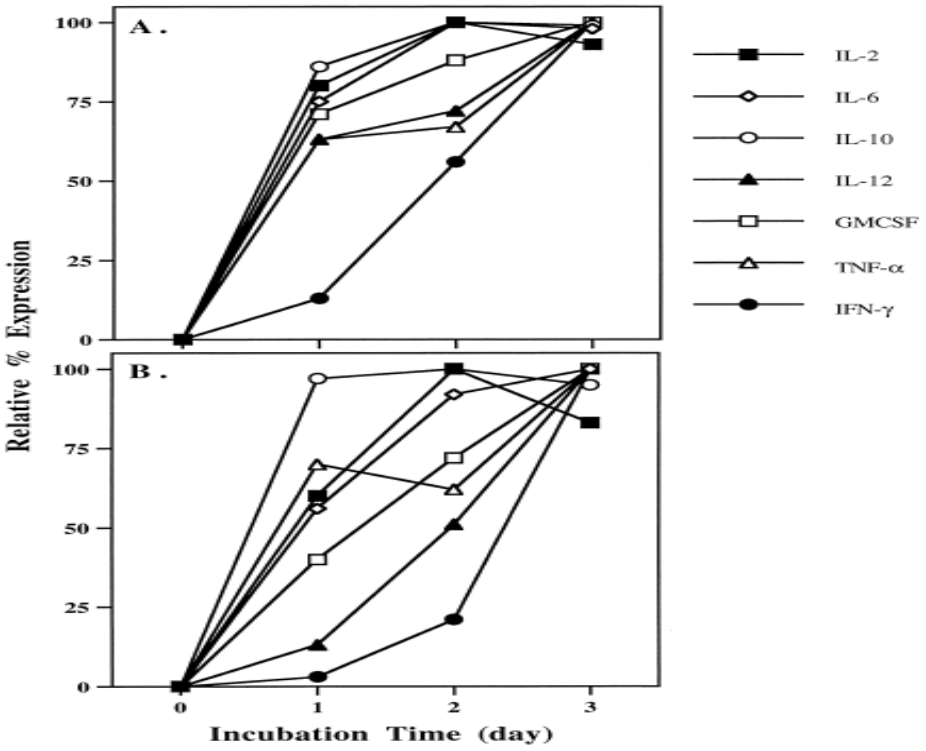

Figure 2.5. Line graph showing the production of cytokines after BCG stimulation in the splenocytes of mice (A) and the PBMCs of humans (B) (Luo, Chen et al. 2003).

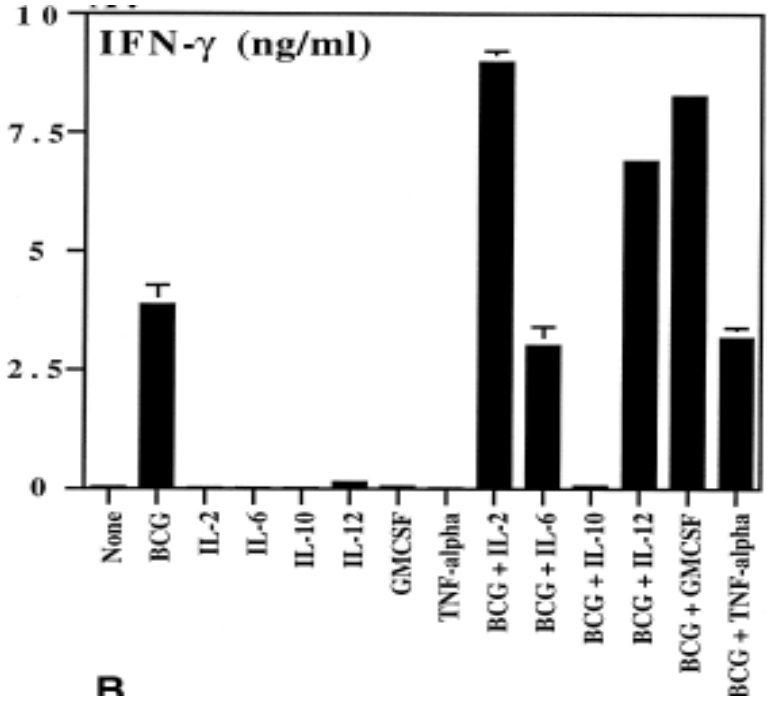

Figure 2.6. Bar graph showing the production of IFN-γ by individual combinations of cytokines with BCG in mouse splenocytes (Luo, Chen et al. 2003).

Most of these cytokines including IL-2, IL-6, IL-10, IL-12, IFN-γ, and TNF-α and GMCSF established significant ranks of IFN-γ in the existence of stimulated BCG (Figure 2.6), but a down-regulation of IFN-γ synthesis was observed in the absence of these cytokines which proves their costimulatory activity for IFN-γ synthesis. However, the presence of the IL-10 cytokine prevented the making of IFN-γ and this is because this cytokine is a contender of Th1 responses and a major Th1 suppressing cytokine. In addition, the Th2 cytokines (IL-6 and IL-10) failed to support BCG for IFN-γ synthesis yielding 76% and 92% inhibition, respectively. Amongst all investigated cytokines, IL-12 was the most potent costimulatory cytokine in BCG-induced IFN-γ production. This can be supported by several of studies that prove the role of IL-12 as a positive upstream regulator of IFN-γ synthesis in NK and Th1 cells, and as the main

initiator of Th1 immune responses (Seder, Gazzinelli et al. 1993; Scharton and Scott 1993; Kobayashi, Fitz et al. 1989). The IL-6 cytokine acts as a multiple purpose cytokine in B cell maturation and in T cell stimulation (Kishimoto, Akira et al. 1995; Kawano, Noma et al. 1995; Taga, Kawanishi et al. 1987). This is verified when nullifying endogenous IL-6 production weakened BCG-induced IFN-γ synthesis in both the splenocytes and PBMCs of mouse and human systems respectively, whilst the addition of exogenous IL-2 inhibited BCG for the synthesis of IFN-γ.

Findings of the analysis of cytokines in BCG-induced IFN-γ production showed that cytokines were not as effective as individuals compared to BCG. However, when rationally combined as individuals or multiples to BCG they demonstrated highly potent effects for IFN-γ production (Table 2.3). Earlier studies have demonstrated the synergistic effect of BCG with either recombinant IFN-α or IL-12 to increase urinary IFN-γ production in human PBMC cultures (O'Donnell, Luo et al. 1999; Luo, Chen et al. 1999). In this similar study Luo, Chen et al.(2003) proved that the synergistic relationship of BCG with cytokines, IL-2, IL-12, IL-18, and GMCSF produced significant amounts of IFN-γ in the splenocytes of mice, whilst on the other hand human PBMCs produced significant amounts of IFN-γ by rational combinations of BCG with cytokines, IL-2, IL-12, TNF-α and IFN-α (figure 5). Furthermore, an analysis of multiply (2 to 3) combined costimulatory cytokines with restricted BCG doses were still able to harvest IFN-γ and surprisingly compensate for the limited dose conditions. These findings suggest the synergistic use of these cytokines with BCG in clinical implications for bladder cancer treatment. Moreover, studies have proven the safe use of purified Th1-stimulating cytokines as immunotherapy of bladder cancer demonstrating marginal antitumor effects (Glazier, Bahnson et al. 1995) (Stricker, Pryor et al. 1996).

Table 2.3: Showing a summary of effect of individual cytokines and rationally combined cytokines to BCG for IFN-γ production (Luo, Chen et al. 2003).

	Single cytokines		**Cytokines plus BCG**	
	Mouse	**Human**	**Mouse**	**Humans**
IL-2	-	+/-	++	+++
IL-6	-	-	-	**
IL-10	-	-	***	***
IL-12	+	+	++	++ ++
IL-18	+	+/-	++ ++	+/-
GMCSF	+/-	+/-	++	-
TNF-a	-	+	-	++
IFN-a	-	+	**	+++

-, no effect; +/-, marginal effect; +, weak effect; ++, mild effect; +++, strong effect, ++ ++, very strong effect; **, strong inhibition, *, very strong inhibition.**

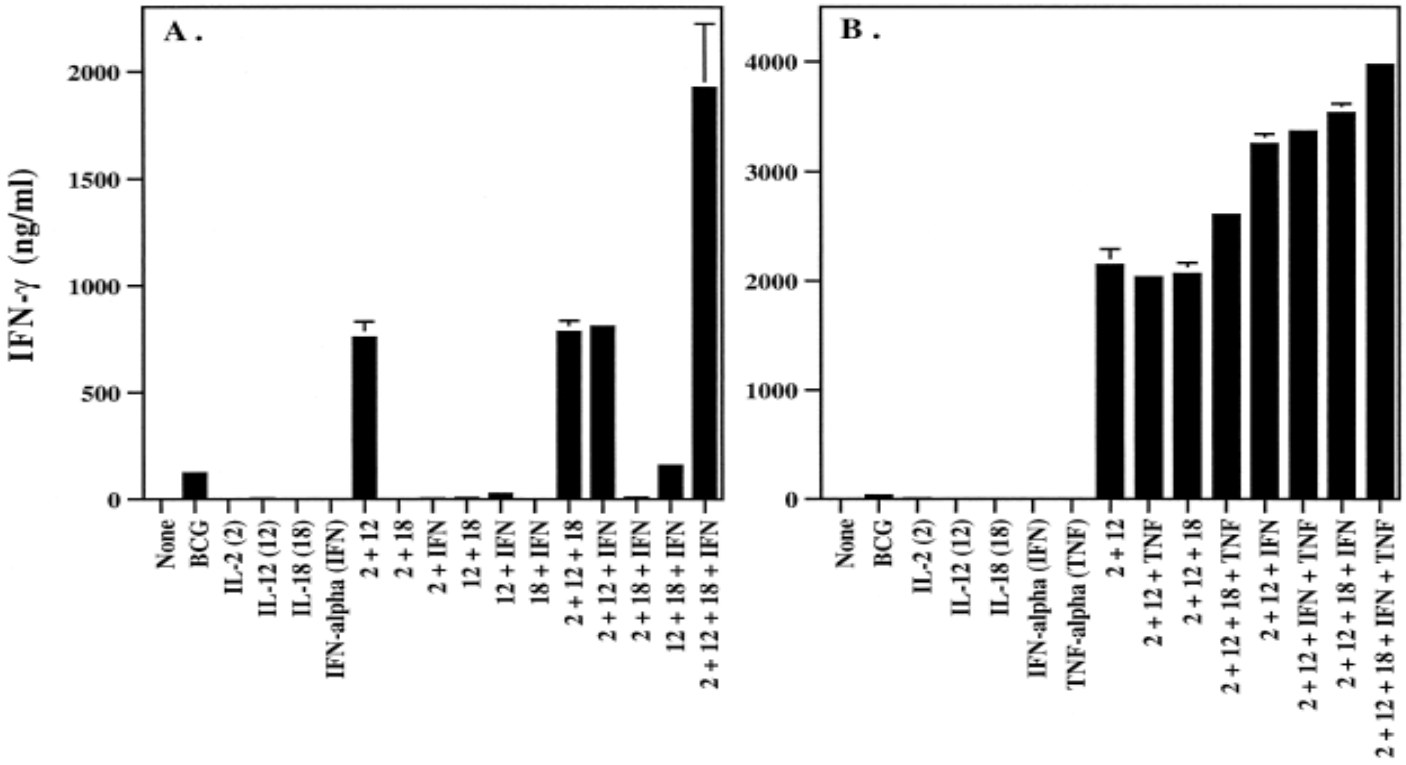

Figure 2.7: Bar graphs showing the rational combination of cytokines for IFN-γ synthesis. The human PBMCs (A) were challenged with $2.5x10^5$ CFU/mL BCG in one of the four displayed recombinant cytokines or multiply combinations, and the effect of incorporating IL-18, TNF-α and IFN-α for IFN-γ synthesis (B) (Luo, Chen et al. 2003).

An interesting effect of particular Th1-stimulating cytokine has been observed by (Luo, Chen et al. 2003). Interestingly, some cytokines have displayed species-specific properties to BCG-induced IFN-γ invention. It has been shown that even though IL-2, IL-6, IL-10, and IL-12 vary in function they display similar effects to BCG-induced IFN-γ production in both mouse and human species. However, IL-18 and GMCSF cytokines inhibited BCG for IFN-γ synthesis in the human species whilst TNF-α and IFN-α inhibited BCG for IFN-γ synthesis in the mice species, and the IFN-α cytokine also did not support BCG for IFN-γ synthesis in mice but supported BCG stimulation in humans for IFN-γ production (Luo et al.2003). This further verifies the species-specific properties of some cytokines to BCG and the inherent differences that exist between the two different species investigated. However, it is also believed that since mice used the splenocytes and the humans used the PBMCs as their source of immune-competent cells, this may have contributed to the role of some of these cytokines.

The synergistic effect of the rationally combined th1-stimulating cytokines for IFN-γ synthesis in the mice and human species was also analyzed (Luo, Chen et al. 2003). Amongst all rational combinations of two cytokines, the IL-2 and IL-12 combination in humans showed the most potent synergistic relationship for IFN-γ production yielding a 6.2-fold increase over BCG (figure 7a) and a 234-fold increase over BCG (figure 7b), compared to the amount of IFN-γ they synthesize when existing as individual components. The production of IFN-γ in this rational combination also supersedes the quantity of IFN-γ produced by each of these cytokines combined to BCG. It is believed that adding Th1-stimulating cytokines such as IFN-α, TNF-α, and IL-18 to these synergistic combinations could promoter higher synergy, thus further IFN-γ production. Hence, these rational combination synergistic effects can be employed in immunotherapy for bladder cancer by promoting urinary IFN-γ synthesis.

In mice, the IL-12 and IL-18 instead of the IL-2 and IL-12 two cytokine rational combination displayed an effective synergistic relationship for the production of IFN-γ. The IL-12 and IL-18 combination demonstrated an 836-fold increase over BCG and a 14-fold increase over BCG. This effect verifies the species-specific properties to BCG-induced IFN-γ synthesis and the different inherent differences of the mouse system to human systems for immune responses. Furthermore, such findings highlight the importance of not always using mice models as representatives of humans, nor their experimental evidence for direct use in clinical applications.

2.8 Interferons (IFNs)

IFNs can be divided into three classes, type I, type II and type III. Type 1 IFNs include IFN-α and IFN-β which are produced by leucocytes and fibroblasts, respectively. These IFNs are synthesized as a result of virally infected cells which in turn trigger factors that aid in disturbing viral DNA or viral RNA replication (Sinigaglia, D'Ambrosio et al. 1999). This early form of host defense is vital in preventing transmission of the infection before more specific immune responses are triggered (Uematsu and Akira 2007). Type II IFNs include IFN-γ and they are mainly synthesized by T cells and NK cells after immune and inflammatory stimulation (Pestka, Krause et al. 2004). Type III mammalian IFNs include IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) (Sheppard, Kindsvogel et al. 2003). However, only one IFN-λ has been found in the chicken chromosome 7 (ChIFN-λ) (Karpala, Morris et al. 2008), and this gene has been revealed to constitute a higher amino acid identity to mammals IFN-λ2 compared to the other IFN-λ individuals, and lower amino acid identity to type I and type II ChIFNs (Karpala, Morris et al. 2008). Karpala, Morris et al. (2008) also observed the difference between ChIFN-λ and ChIFN-γ in producing NO, and found that both genes enhance the production of NO but ChIFN-λ synthesized smaller quantities

than ChIFN-γ. Moreover, the inhibition of viral activity against Semiliki Forest Virus (SFV) and influenza virus by both these genes has been observed comparable to that of type I ChIFNs (Karpala, Morris et al. 2008).

2.8.1 Type II IFN (IFN-γ)

The type II IFN (IFN-γ) is a pleiotropic cytokine produced by countless sorts of immune cells under inflammatory conditions, particularly T cells and NK cells. It plays an imperative role in both the innate and adaptive immune system by stimulating macrophages and encouraging the activity of TH1 cells which leads to improved phagocytosis, improved MHC class I and class II expression, and the initiation of IL-12, NO, and superoxide formation. All these biological activities assist in eradicating intracellular viruses (Pestka, Krause et al. 2004). Furthermore, IFN-γ binds to a receptor complex designated as IFN-γ-R which is made up of two receptor chains (R1 and R2), and this complex-receptor interaction permits the activation of the signal transduction pathway by employing the Janus activating kinase (JAK) or the signal transducer activator of transcription (STAT). The signalling of this pathway occurs when a signal is transmitted to the cell's nucleus for transcription of the IFN-γ induced genes. The IFN-γ encourages the generation of anti-viral proteins, such as reactive nitrogen intermediates and protein kinase R from monocytes and macrophages (Malmgaard 2004), which then leads to the cytotoxic kill of various intracellular pathogens. Scientists observed and reported the role of IFN-γ in immunity, whereby mice in good conditions were challenged with sub-lethal doses of viral and bacterial infection; although the mice used were in good health they lacked the IFN-γ, IFNγ-R1 and IFNγ-R2 genes resulting to their death (Huang, Hendriks et al. 1993). The synergistic relationship between IL-12 and IL-18 are known to produce great quantities of IFN-γ compared to the quantity produced by each cytokine on its own. This cytokine interaction helps to generate high levels of cytokines in cell populations, such as macrophages (Munder, Mallo et al. 1998), dendritic cells (Fukao, Frucht et al. 2001), $CD4^{*}$ (Kohno, Kataoka et al. 1997), and CD8* (Tomura, Maruo et al. 1998) T cells irrespective of TH1 cell differentiation (TCR) stimulation or inhibition.

2.8.2 Chicken interferon gamma (ChIFN-γ)

All mammalian and avian species populations have been reported to possess a well-maintained function of the IFN-γ gene. The chIFN-γ gene is imperative for the functioning of both the anti-viral and macrophage-activating factor, augmenting RNA production of guanylate-binding proteins, and potentially increases the expression of MHC class II genes and triggers the release of NO in macrophages (Lowenthal, York et al. 1998). Moreover, the gene essentially interferes with the replication of cytolytic viruses in chicken embryo fibroblasts and escalates resistance to coccidiosis (LOWENTHAL, YORK et al. 1997) and *E.coli* infection.

2.8.3 The functional role of interferons

The immunological function of IFN-γ has been demonstrated in various diseases in poultry such as, Newcastle disease virus (Brown, King et al. 1999), *S. enteritidis* and *Brucella abortus* (BA) (Zhou, Lillehoj et al. 2002), *Eimeria tenella* and *Eimeria acervulina* (Lillehoj and Choi 1998) and Rous Sarcoma virus (RSV) (Plachý, Weining et al. 1999). The human IFN-γ genes have shown sequence polymorphisms that confer resistance to disease (Awata, Matsumoto et al. 1994). Chickens are often said to be robust and fit due to their ability to express high levels of antibodies against pathogenic bacteria such as, *Pasteurella multicoda* (*P. multicoda*), thus disease resistant (Hofacre, Glisson et al. 1987) . Zhou, Buitenhuis et al. (2001) has previously reported the role of the mutation at the IFN-γ promoter region to be meaningfully connected with increased primary and secondary antibody titers in chicken against SRBC and *Brucella abortus* (*BA*) antigens (Zhou, Buitenhuis et al. 2001). Hence, this suggests the functional activity of the IFN-γ promoter polymorphism in disease resistance. Additionally, IFN-γ essentially protects the host by provoking intestinal immunity against Salmonella bacteria such as S. typhimurium in the mouse (Bao, Beagley et al. 2000). The expression of IFN-γ mRNA and protein is chiefly portrayed by T cells and large granular lymphocytes. Lymphocytes can only express IFN-γ by the co-operative functioning of accessory cells such as mononuclear phagocytes (Billiau 1996).

Zhou and Lamont (2002) investigated the association of circulating IFN-γ levels with the nucleotide substitutions of the IFN-γ promoter and antibody response, so as to analyze the probable of the IFN-γ protein in facilitating genetic control of antibody response in chickens. A significant association between antibody response to SE and circulating IFN-γ protein level at both post-primary and post-secondary periods were reported suggesting that humoral immunity can be induced by genetic selection of optimal IFN-γ genotypes (Zhou, Lillehoj et al. 2002). In the study, they also predicted that the IFN-γ promoter

mutation would affect IFN-γ gene expression since the promoter region of the gene controls gene expression. Findings demonstrated consistent allelic effect results on IFN-γ protein level. The leghorn (LL) genotype of IFN-γ persistently showed the highest expression of antibodies compared to the LF (heterozygote of both leghorn and Fayoumi alleles) and the homozygote from the Fayoumi genotype (FF); after both primary and secondary immunization and in F_2 offspring of both M5.1 and M15.2 grandsires (Zhou, Lillehoj et al. 2002). Furthermore, they reported strong variations of the grandsire lineages, a 15.8% of phenotypic variation on IFN-γ protein level at 7 days after primary immunization in F_2 offspring of the M15.2 lineage, and an 8.5% for IFN-γ synthesis at 7 days after secondary immunization of the M5.1 grandsire. These findings propose that DNA sequence polymorphisms occurring in or around the IFN-γ promoter influence IFN-γ protein level after both immunizations. Non-significant associations of IFN-γ protein with primary antibody response to both SRBC and BA after secondary immunization were reported. However, a noteworthy correlation was observed of IFN-γ protein with secondary antibody response to SRBC after both primary and secondary immunizations and negative associations with the time needed to establish secondary antibody response to SRBC. Hence, a directly proportional relationship exists between induced levels of IFN-γ protein and induced maximum secondary antibody response to SRBC (Zhou, Lillehoj et al. 2002). Therefore, the exogenous application of IFN-γ may be an effective approach to induce antibody response to promoter vaccinations. The expression IFN-γ induces NO production from activated macrophages in order to inactivate Salmonella in the caecum (Rychlik, Elsheimer-Matulova et al. 2014). Pieper also confirms the necessity of IFN-γ in stimulating bactericidal activities of macrophages against *Salmonella Typhimurium* (*S. typhimurium*) (Pieper, Methner et al. 2011). Many immune relevant cytokines including IFN-γ have been reported to be highly expressed and produced within a 48-hour post-infection period of chickens infected with Salmonella (Crhanova, Hradecka et al. 2011; Rychlik, Elsheimer-Matulova et al. 2014). IFN-γ is estimated to be fully expressed around 4-days post-infection. The expression of IFN-γ simultaneously occurs with that of TGM3, TGM4, PRG1, TRAP6, ExFABP, iNOS, AVD, and SERPINB10 effector proteins (Matulova, Havlickova et al. 2013). Thereafter, IgY and IgA expression follows in the gut lumen of infected chicken which aids in removing Salmonella (Matulova, Havlickova et al. 2013). It has also been reported that induced quantities of $CD8_{\alpha\beta}^{+}$ γδ and $CD8_{\alpha}\beta^{+}$ T cells in the blood and spleen of chickens help to activate and induce the expression of Th1 proteins, such as Fas, FasL, IL-$2R_{\alpha}$ and IFN-γ after S. typhimurium infection of young chicks (Pieper, Methner et al. 2011).

The MHC of chickens is also known to assist in the synthesis of antibodies against pathogenic bacteria. Lamont (1998) highlighted that high-antibody production lines confer greater resistance capabilities to parasites and viruses; however not bacteria when likened to little-antibody production lines. (Leitner, Melamed et al. (1990) and Yonash, Heller et al. (2000) also demonstrated correlation of increased

antibody counts with resistance to pathogenic bacteria such as, *E. coli* and *S. enteritidis*. However, since a variety of components of immune response participate in resistance to diseases, the association between SE antibodies and response to live SE challenge had to be evaluated by determining the genetic relationship between SE colonization and SE vaccine antibody response in young broiler breeds. In the study, half-sib groups of chicks were challenged with live SE or vaccinated against SE. The ELISA method was used to determine the serum antibodies at 10-day post-vaccination whilst the SE counts were analyzed at 21 or 42 weeks of exposure to SE. Findings showed a negative correlation (-0.87) of antibody response to vaccination and the number of SE in caecal swab cultures. Hence, the genetic potential for greater SE vaccine antibody response was allied with lower SE caecal colonization in broiler breeds challenged with live SE. Moreover, these findings suggest the use of genetic selection for improving response to SE colonization by selecting for increased SE antibody response. The above mentioned information proves that when particular cytokine genes are in synergistic collaborations; more effective and powerful immune responses are demonstrated and as individuals, both the IL-2 and IFN-γ cytokine genes have demonstrated their potential ability to induce genetic resistance against particular diseases and the reduction of bacterial burden loads in tissue organs of avian species.

Conclusion

Several studies have focused on the genetic potential of commercial chickens to fight off infections, and their ability to produce more than sufficient poultry products (eggs and meat) compared to indigenous breeds under the commercial poultry production system. However, there is a gap of information on the genetic potential of indigenous chickens living in extensive or semi-intensive systems. Therefore, this study aims at genotyping for cytokine genes, particularly IL-2 and IFN-γ for the identification of SNPs that may be responsible for disease resistance. The background of this review highlighted the famous nature (robustness and fitness traits) of indigenous chickens in extreme environmental conditions. Hence, the null hypothesis of this study is that indigenous chickens will demonstrate a higher genetic diversity compared to commercial chickens, due to them possessing better robustness and fitness traits such as, their ability to survive on small-holder low-input systems and harsh environmental conditions. Therefore, it is key to gauge the population-genetic parameters of the genes in question in South African indigenous chickens for proper analysis of these traits.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of blood samples from South African indigenous chickens

Study Site

One-hundred (100) indigenous chickens were randomly sampled from different locations in KwaZulu-Natal, South Africa as depicted in Figure 3.1 for the purposes of this study. Out of the 100 blood samples collected, sixty samples came from Pietermaritzburg (29˚31′43″S 30˚15′56″E) whilst forty-five and five came from Kwamashu (29˚45′03″S 30˚57′43″E) and Victoria Market (-29.8560˚S 31.0262˚E), respectively. The indigenous chickens consisted of each of the following four different indigenous chicken breeds found in South Africa: Potchefstroom Koekoek, Venda, Ovambo and Naked Neck as previously described by (Grobbelaar, Sutherland et al. 2010). There were also several instances in which the actual breed could not be discerned due to hybridization amongst several indigenous breeds because in most cases the indigenous chickens are reared without a proper or structured breeding plan in South African local communities.

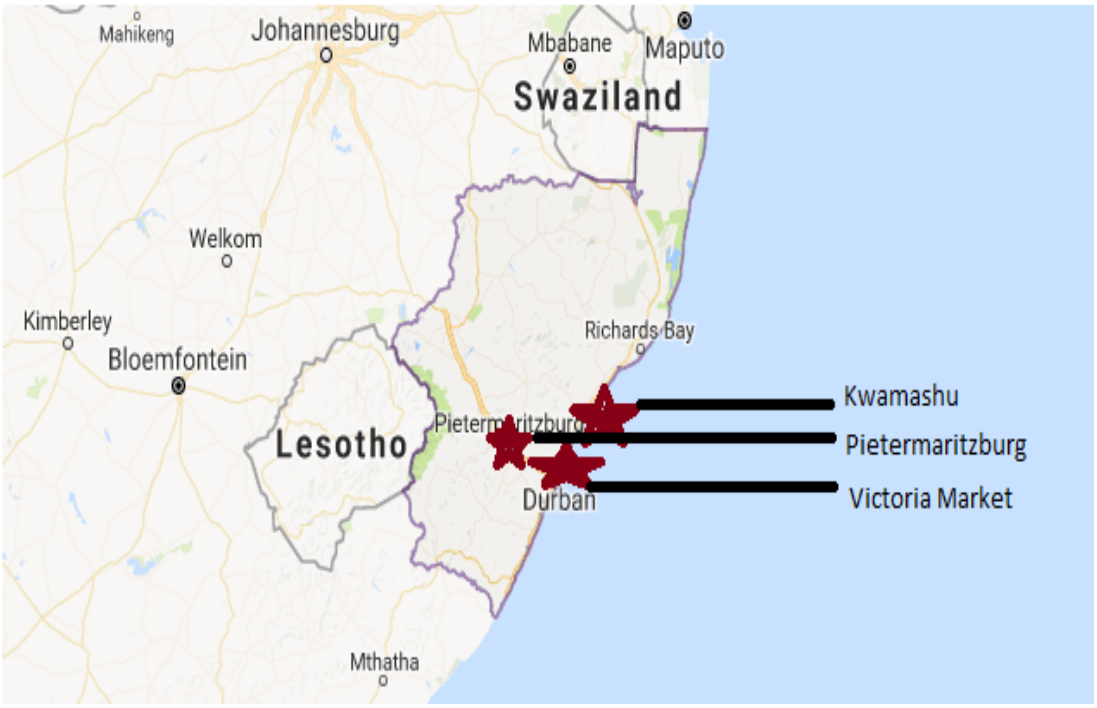

Figure 3.1: Map of Kwazulu-Natal showing the three different areas from which chickens were sampled. The red stars highlight the areas of sampling.

Blood sampling

The wing vein bleeding technique was used for collection of the blood samples. A swab dipped in 70% alcohol was used to disinfect the area from which the blood was to be drawn. Then, a 4.0 mL syringe was used to gently draw blood from a pronator muscle called a tendon, which lies across the wing vein (which is a vein that runs between the biceps and triceps muscles, forming a V-shape), and Thereafter, pressure was applied to the vein to prevent further bleeding. Some of the chickens were slaughter on their necks, thus blood collection occurred from the necks. All collected blood samples were aseptically transferred into 4mL EDTA tubes and anti-coaggulant, or immediately spotted on Whatmann FTA Micro^{TM} cards.

DNA extraction

Genomic DNA was extracted from the blood samples using the phenol-chloroform extraction method. This method included two phases, phenol-chloroform (phase 1) and ethanol precipitation (phase 2). In phase 1, an equal volume of phenol-chloroform was added to the blood sample and centrifuged to form an aqueous layer (Top layer with DNA) and a bottom layer (phenol-chloroform liquid waste). The aqueous

layer was transferred into a new tube with an equal volume of chloroform, whilst the bottom layer was discarded. In phase two, 0.3mg of sodium acetate and two volumes of 100% ethanol were added to the aqueous layer and centrifuged to remove the supernatant. The DNA (which remained as a pellet at the bottom of the tube) was further washed by an addition of 70% ethanol filling up the tube, removing any remains of supernatant. The pellet was left to dry upside down on a rack for 30 minutes. The extracted genomic DNA was then eluted in TE buffer (Tris mM and EDTA mM at pH 7.0), and the quality and concentration of the DNA was confirmed using the Nanodrop™ and by performing agarose gel electrophoresis.

3.2 Screening for disease resistance and susceptibility genes

All polymerase chain reactions were performed in a 25µL master mix reaction, containing 100ng of genomic DNA, and the amplification conditions were as follows: initial denaturation at 95°C for 5min, followed by 40 cycles of denaturation at 95°C for 45s, annealing at a gradient of 64 to 54°C for 45s as shown in Table 3.1, with an extension step at 72°C for 1min, and a final extension at 72°C for 10min.

Table 3.1: Primer sets and annealing temperatures used for the PCR-RFLP assay of the IFN-γ and IL-2 genes

Gene	Accession no.	Primer Sequence	PCR Product (bp)	Annealing Temperature (°C)/time	Reference
IFN-γ	Y079221	F':5'GTAAGGAACTTCAGCCATTG-3' R':5'GACGAATGAACTTCATCTGCC-3'	670	64-54/45s	Zhou et al. (2003)
IL-2	AJ224516	F': 5'-TGCTTTTAACCGTCTTTG-3' R':5'-GATGCTCCATAAGCTGTAGT-3'	659	64-54/45s	Zhou et al. (2003)

The PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method was used to genotype for SNPs present in both the IL-2 and IFN-γ candidate genes. The set of primers used for amplifying the fragments of these genes have been previously described in other studies (Table 3.2).

Thermo Scientific fast digest restriction enzymes were used to screen for polymorphisms of candidate genes. The PCR products of IL-2 and IFN-γ were digested using *MnlI* (5U) at 37°C and *TasI* (2U) at 65°C respectively, for 5 to 15min. The digested products were confirmed by running a 3% Agarose gel with ethidium bromide staining for 2h at 80V and visualized under ultraviolet light. This procedure enabled the assignment of genotypes to each chicken for each gene.

Table 3.2: Conditions and RE's used for genotyping in IL-2 and IFN-γ genes, and their exact SNP location.

Gene	SNP location	Restriction enzyme	Incubation temp. (°C)	Incubation duration (min)	Reference
IFN-γ	-318bp of 5' flanking region (A → G)	TasI	65°C	5-15	Tohidi et al. (2012)
IL-2	-425bp of 5' flanking region (A → G)	MnlI	37°C	5-15	Tohidi et al. (2012)

3.3 Agarose gel electrophoresis

Loading buffer

A mixture containing 0.01g of bromophenol blue and 1.5mL of 80% glycerol was made which was then totaled up to 4mL with distilled water (dH_20). Thereafter, 3µL of Gel red™ was added to the loading buffer.

10X TBE

A 10X TBE stock solution was made by diluting the following reagents: 108g Tris, 55g Boric acid, 40mL or 9.3g Na_2EDTA (0.5M) with pH 8.0, and 800mL dH_20. The final volume of the solution was 1 litre.

10X TAE

A 10X TAE stock solution was made by diluting the following reagents: 8.4 g of Tris, 11.4 mL of glacial acetic acid (17.4 M), 3.7 g of EDTA, disodium salt, and 800mL de-ionized water. The final volume of the solution was 1 litre.

TE Buffer

To make a 100mL of TE buffer, the following reagents were diluted: 1mL of 1M Tris-HCl (pH 8.0) and 0.2mL EDTA (0.5M) and distilled water up to 100mL.

Agarose gel

The genomic DNA agarose gels were prepared by weighing and dissolving 0.9g of agarose powder in 100mL of 1X TBE to make a 0.9% (w/v) gel which was microwaved for 1 to 2 minutes. On the other hand, the PCR gels were prepared by weighing and dissolving 3g of agarose powder to make a 3.0% (w/v) gel, which was heated for 2 minutes. Once cooled off, the prepared gel solution was poured into a casting tray to solidify for 30 minutes.

3.4 Statistical analysis

A statistical analysis was performed by taking population-genetic measurements such as, the Inbreeding co-efficient (F_{IS}), the Shannon Fixation Index (I) (Lewontin 1972), allele and genotypic frequencies, N_{ei}, Chi square (χ^2), observed and expected homozygosity and heterozygosity (Levene 1949), observed and the effective number of alleles, and the Hardy-Weinberg Equilibrium (HWE). The PopGene software version 1.31 was used to calculate all the above mentioned genetic measurements (Yeh and Yang 2000).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 PCR results

The extracted Genomic DNA was amplified using the polymerase chain reaction, and amplification was confirmed by running a 0.9% Agarose gel at 80V for 1hr. The amplified PCR products for the IL-2 and IFN-γ gene were 659bp (figure 4.1) and 670bp (figure 4.2), respectively. The sizes of the PCR products were measured using a 100bp molecular weight marker.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

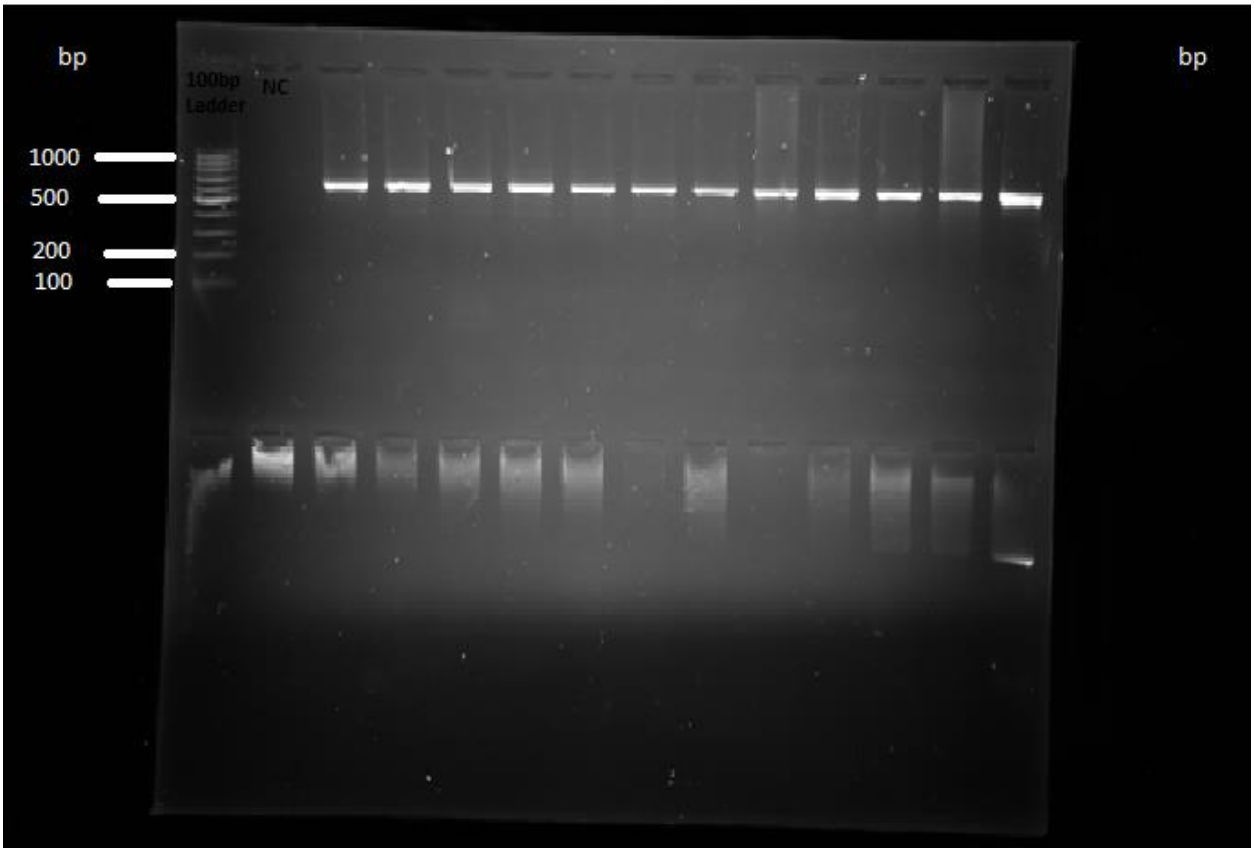

Figure 4.1: A 0.9% Agarose gel image showing the 659bp amplified PCR product of IL-2 from lane 3 to 14, run for 1hr at 80V**.**

1 2 3 4 5 6 7 8 9 10 11 12 13 14

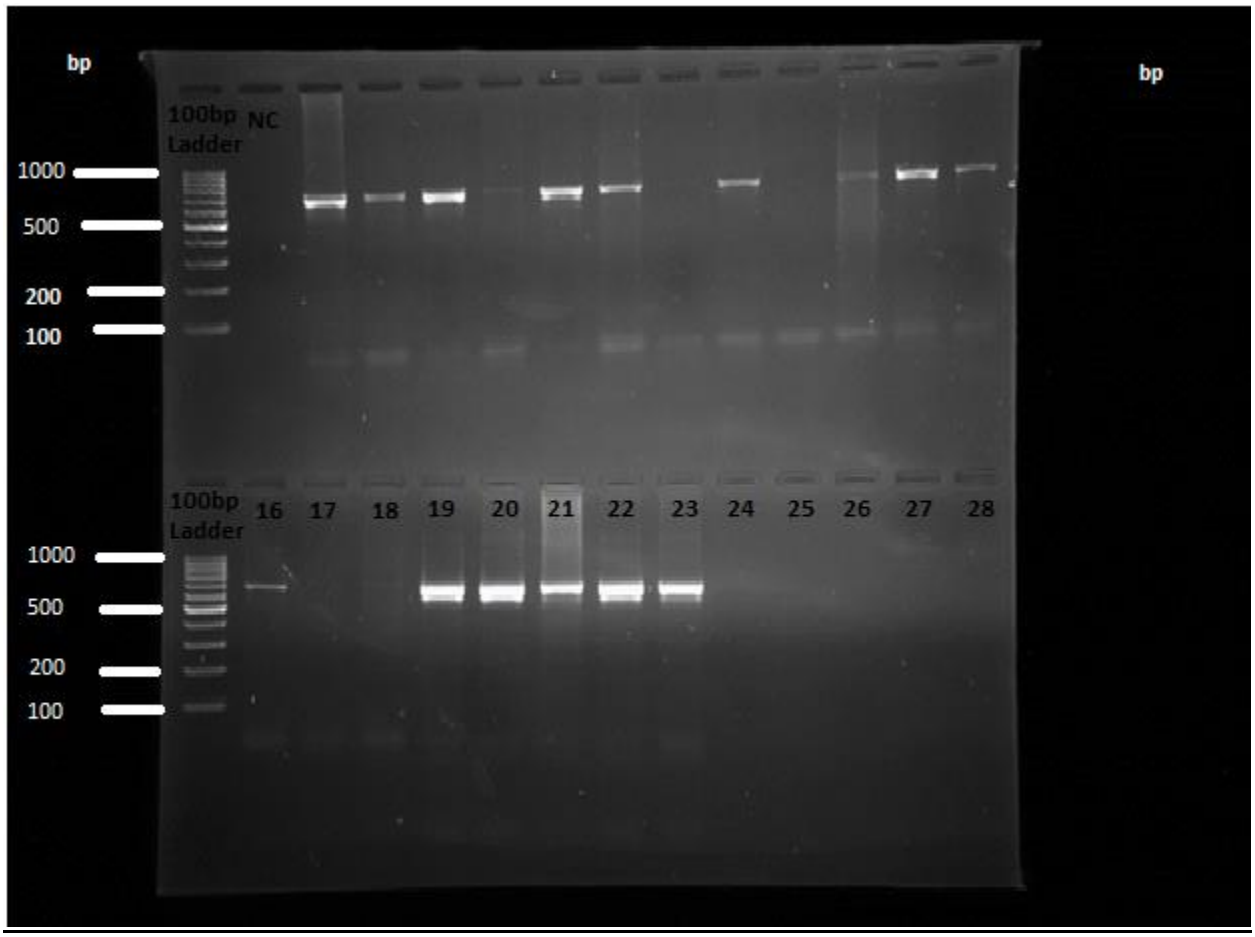

Figure 4.2: A 0.9% Agarose gel image showing the 670bp amplified PCR product of IFN-γ from lane 3 to 14 and 16 to 28, and a negative control (NC) in lane 2 and 16 run for 1hr at 80V

4.2 RFLP results

Polymorphisms of candidate genes were screened using restriction enzymes, and the IL-2 gene was digested with the *MnlI* RE. The promoter region of this gene had an A/G substitution at -318bp, which produced a different digest pattern of the 659bp PCR product (Figure 4.3). Common fragments of 123, 130, and 156 were generated for the G-allele, and an extra 250bp fragment was produced for the A-allele. The heterozygotes (AG) had a combination of all the four bands. The allele frequencies for both alleles A and G of this gene were 0.5476 and 0.4524, respectively.

1 2 3 4 5 6 7 8

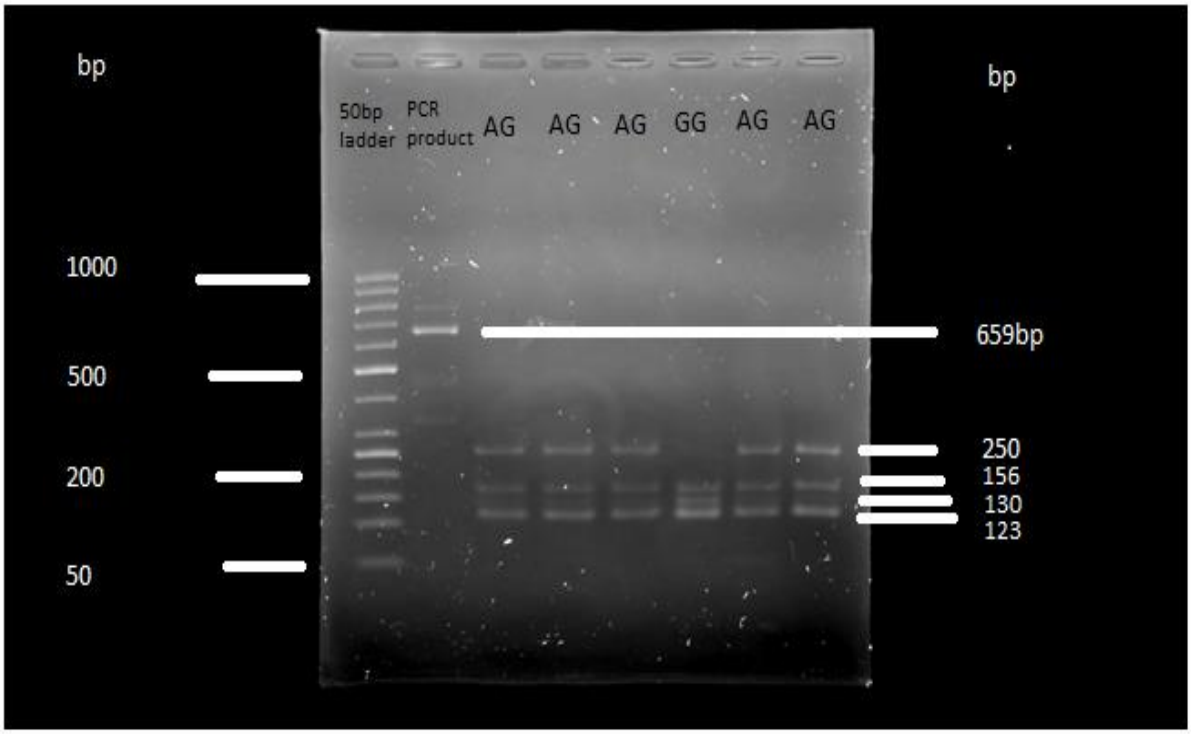

Figure 4.3: Image of a 2.5% Agarose gel showing PCR-RFLP fragments of the IL-2 gene after nuclease activity by the *MnlI* restriction enzyme

Nuclease activity by the *TasI* restriction enzyme produced the following common fragments of the IFN-γ gene: 55; 56; 99; 100; 123; and 168bp for the G-allele and an extra 69bp fragment for the A-allele (figure 4.4). The allele frequency for both alleles A and G were 0.1579 and 0.8421, respectively.

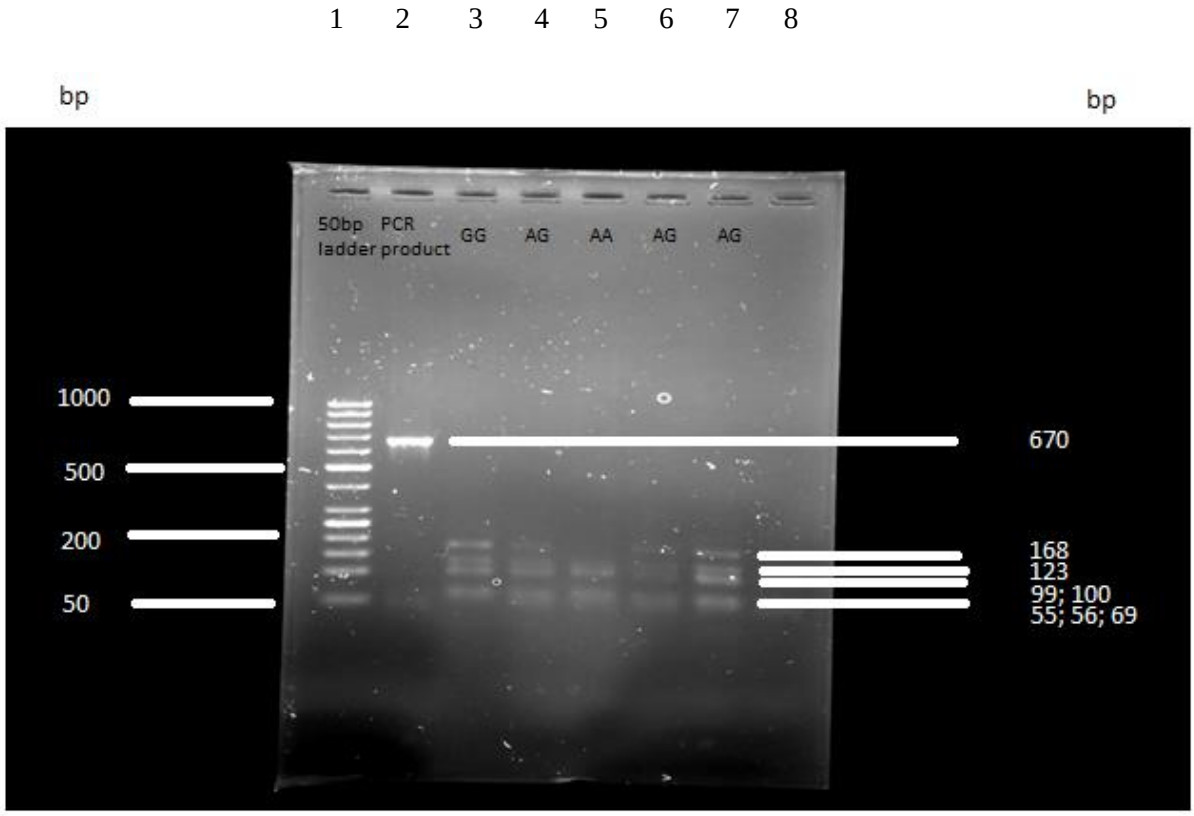

Figure 4.4: A 2.5% Agarose gel showing PCR-RFLP fragments of IFN-γ after nuclease activity by the *TasI* restriction enzyme

4.3 Population genetic parameters results

According to 'Shane's simple guide on f-statistics,' the Shannon Fixation Index (I) value measures the extent of genetic differentiation among sub-populations, and this value usually ranges between zero (indicating lack of differentiation) and one (indicating complete differentiation among sub-populations) (Weir and Cockerham 1984). In PMB, the IL-2 locus had an I-value of 0.6886 whilst the IFN-γ I-value was 0.4362 (Table 4.1). This means that there was 68.9% and 43.6% of genetic differentiation amongst sub-populations and 31.1% and 56.4% of genetic differentiation within sub-populations for the IL-2 and IFN-γ loci, respectively. However, the IL-2 locus had the greatest genetic differentiation due to increased levels of genetic drift among sub-populations and most probably small population size. Under the influence of genetic drift, sub-populations diverge genetically and migration limits genetic divergence, thus keeping the sub-populations similar (Hartl, Clark et al. 1997).

Table 4.1: Showing the observed number of alleles (na^*) and effective alleles (ne^-) of both the IL-2 and IFN-γ loci in Pietermaritzburg (PMB).

Locus	Sample size	Na	Ne	I^*
IL-2	42	2.0000	1.9820	0.6886
IFN-γ	38	2.0000	1.3623	0.4362
Mean	40	2.0000	1.6721	0.5624
Standard deviation	0.0000	0.4382	0.1785	

*na = the observed no. of alleles.

*ne = the effective no. of alleles (Kimura and Crow 1964).

*I = Shannon's Information Index (Lewontin 1972).

The PMB population demonstrated 47.62% and 52.38% of observed homozygosity and observed heterozygosity for the IL-2 locus respectively, whilst the IFN-γ locus showed 100% and 0% of observed homozygosity and observed heterozygosity, respectively (Table 4.2). This shows that the IFN-γ locus lacked heterozygotes within the population and this is seen by complete inbreeding, thus no genetic variability was observed in the population. Subdivisions within the PMB population may have also caused the loss of genetic variability within sub-populations as a result of small populations and genetic drift acting within each one of them (Wright 1965). Genetic drift within a small population size and chicken type is the greatest influence to the genetic background of a chicken (Nassiry, Javanmard et al. 2009). The explanation of excess homozygotes and deficiency of heterozygotes within the Cedara population for the IFN-γ locus was previously described as an influence of inbreeding and low fitness by (Keller and Waller 2002). However, the IL-2 locus demonstrated greater genetic variability compared to the IFN-γ locus as it had more heterozygotes than homozygotes present within the PMB population, thus more levels of outbreeding due to random mating. Although the IL-2 locus had greater variability, it also had a fair share of homozygotes and heterozygotes in the population. Hence, fair levels of inbreeding and outbreeding. It is said that He lower than 0.5 is maximum heterozygosity at a bi-allelic locus which is the case for both loci within PMB (Dorji, Daungjinda et al. 2011; Akaboot, Duangjinda et al. 2012). The Nei values of both loci (IL-2, 0.4955; IFN-γ, 0.2731) were documented via (Nei 1973) and these indicated a low level of heterozygosity, although that of IL-2 is greater than that of IFN-γ.

Table 4.2: Observed and expected homozygosity and heterozygosity of IL-2 and IFN-γ loci within PMB.

Locus	**Sample size**	**Observed homozygosity**	**Observed heterozygosity**	**Expected homozygosity**	**Expected heterozygosity**	**Nei**	**Avg. heterozygosity**
IL-2	42	0.4762	0.5238	0.4925	0.5075	0.4955	0.4383
IFN-γ	38	1.0000	0.0000	0.7269	0.2731	0.2659	0.3386
Mean	40	0.7381	0.2619	0.6097	0.3807	0.3885	
St. deviation	0.3704	0.3704	0.1658	0.1658	0.1623	0.0705	

*analysis of expected homozygosity and heterozygosity were computed via (Levene 1949).

*Nei's expected heterozygosity was documented via (Nei 1973).

The inbreeding co-efficient (F_{IS}) is a measure of heterozygote deficiency or excess within a sub-population due to genetic inbreeding or outbreeding. The F_{IS} for both alleles A and G for the IL-2 locus at PMB was -0.0572, whilst that of IFN-γ was 1.0000 (Table 4.3). These findings indicate that there was very little genetic inbreeding within a sub-population for the IL-2 locus and high outbreeding levels as there was a great amount of heterozygotes compared to homozygotes. Increased outbreeding levels are usually an indication of fitness within sub-populations (Keller and Waller 2002). The IFN-γ locus demonstrated complete inbreeding within a sub-population due to no observed heterozygosity and excess homozygosity (Table 4.3), thus no genetic trace of variability. Upon non-random mating, the equilibrium of allelic frequencies may have been disturbed due to the presence of mutational changes within the PMB population, whilst disturbances such as natural selection and genetic drift may have interfered with genotype frequencies thus a possible reason for the Hardy-Weinberg Equilibrium (HWE) disturbance of the IFN-γ locus.

Table 4.3: The measure of heterozygote deficiency or excess in PMB

Allele/locus	**IL-2**	**IFN-γ**
A	-0.0572	1.0000
G	-0.0572	1.0000
Total	-0.0572	1.0000

The I-value of both the IL-2 and IFN-γ loci for Kwamashu was 0.5623, meaning that 56.23% of genetic differentiation existed among the sub-populations with 43.77% having had existed within sub-populations (Table 4.4). Thus, low genetic variability occurred within the Kwamashu population for both loci. A fair level of genetic drift occurred among sub-populations with limited genetic divergence as a result of migration (Wright 1965). The IL-2 and IFN-γ loci of the Kwamashu population showed a number of 2 and 1.6 observed and effective number of alleles, respectively. This indicates that 0.4 (difference of the observed and effective no. of alleles) was ineffective and was either null or private alleles. Hence, although mutations may significantly change a gene pool, most are considered neutral or deleterious (Glasenapp, Frieden et al. 2015) (Fang, Larson et al. 2009).

Table 4.4: The observed number of alleles (na*) and effective alleles (ne*) of both the IL-2 and IFN-γ loci in Kwamashu.

Locus	Sample size	Na	Ne	I*
IL-2	20	2.0000	1.6000	0.5623
IFN-γ	32	2.0000	1.6000	0.5623
Mean	26	2.0000	1.6000	0.5623
Standard deviation	0.0000	0.0000	0.0000	

The observed and expected homozygosity and heterozygosity of both IL-2 and IFN-γ were analyzed in the Kwamashu population. Both these loci showed high-observed genetic similarities compared to the expected. There was 90% and 100% of observed homozygosity within sub-populations for the IL-2 and IFN-γ loci respectively, and 10% and 0% of observed heterozygosity within sub-populations for each of the loci, respectively (Table 4.5). This indicates very low genetic variability within the sub-populations of the IL-2 locus, having the IFN-γ locus show no variability at all due to small population size and genetic drift acting within each one of them. Upon genetic drift, coincidental incorporation of a new mutant allele occurs throughout the entire population for "new" genetic variation and all prevailing molecular polymorphism (Glasenapp, Frieden et al. 2015). The Nei value for both loci was 0.3750, indicating substantially low levels of heterozygosity within the Kwamashu population.

Table 4.5: Observed and expected homozygosity and heterozygosity of IL-2 and IFN-γ loci within Kwamashu.

Locus	Sample size	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	Nei	Avg. heterozygosity
IL-2	20	0.9000	0.1000	0.6053	0.3947	0.3750	0.4383
IFN-γ	32	1.0000	0.0000	0.6129	0.3871	0.3750	0.3386
Mean	26	0.9500	0.0500	0.6091	0.3909	0.3750	0.3885
St. deviation	0.0707	0.0707	0.0054	0.0054	0.0054	0.0000	0.0705

The F_{IS} value for both alleles A and G of the IL-2 and IFN-γ loci in the Kwamashu population were 0.7333 and 1.000, respectively (Table 4.6). This means that the extent of genetic inbreeding within sub-populations were 73.33% and 100% for IL-2 and IFN-γ, respectively. However, the IFN-γ locus demonstrated the highest and complete inbreeding compared to IL-2, as a result of extreme heterozygote deficiency or extreme subdivision as a result of complete isolation of the population (Weir and Cockerham 1984). Hence, increased levels of inbreeding lead a sub-population to deviate from the HWE, because upon non-random mating disruptive changes such as mutations, natural selection, genetic drift and gene flow can disturb the HWE. Mutational events can incorporate new alleles within a sub-population that lead to inconsistencies of allele frequencies, whilst a genetic drift interferes with the genotype frequencies of the IL-2 locus within this sub-population.

Table 4.6: The measure of heterozygote deficiency or excess in Kwamashu.

Allele/locus	IL-2	IFN-γ
A	0.7333	1.0000
G	0.7333	1.0000
Total	0.7333	1.0000

In the Victoria Market population, the I-values of the IL-2 and IFN-γ loci were 0.6365 and 0.5623, respectively (Table 4.7). The extent of genetic differentiation was 63.65% and 56.23% amongst sub-populations, and 36.35% and 43.77% within sub-populations for the IL-2 and IFN-γ loci, respectively. This confirms low levels of genetic variability in both these loci within sub-populations due to small population size and genetic drift acting within each one of them. The difference between the number of observed alleles and the effective number of alleles for the IL-2 and IFN-γ loci were 0.2 and 0.4, respectively. This means that there was 0.2 and 0.4 number of private or null alleles present within a sub-population of the IL-2 and IFN-γ loci respectively.

Table 4.7: The Shannon Fixation Index, and the observed number of alleles (na^*) and effective alleles (ne^-) of both the IL-2 and IFN-γ loci in Victoria Market.

Locus	**Sample size**	**Na**	**Ne**	**I***
IL-2	6	2.0000	1.8000	0.6365
IFN-γ	4	2.0000	1.6000	0.5623
Mean	5	2.0000	1.7000	0.5994
Standard deviation		0.0000	0.1414	0.0525

The observed homozygosity and heterozygosity of both the IL-2 and IFN-γ loci in Victoria Market were 100% and 50% respectively within sub-populations (Table 4.8). Both these loci showed observed homozygosity to be greater than the expected homozygosity. The IL-2 locus showed no genetic variability due to excessive homozygotes in the population, indicating complete inbreeding within its sub-population, thus low fitness in this population (Keller and Waller 2002). IFN-γ showed a fair distribution of homozygotes and heterozygotes in Victoria Market, meaning that a fair level of outbreeding and inbreeding occurred within the sub-populations. The Nei value reflected low level of heterozygotes for both loci (IL-2, 44.44%; IFN-γ, 37.5%) in the Victoria Market population (Table 4.8).

Table 4.8: Observed and expected homozygosity and heterozygosity of IL-2 and IFN-γ loci within Victoria Market.

Locus	Sample size	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	Nei	Avg. heterozygosity
IL-2	6	1.0000	0.0000	0.4667	0.5333	0.4444	0.4383
IFN-γ	4	0.5000	0.5000	0.5000	0.5000	0.3750	0.3386
Mean	5	0.7500	0.2500	0.4833	0.5167	0.4097	0.3885
St. deviation		0.3536	0.3536	0.0236	0.0236	0.0491	0.0705

The F_{IS} value for both alleles A and G of IL-2 for Victoria Market was 1.0000 (Table 4.9), indicating the absence of heterozygotes and complete isolation due to extreme subdivision within the population. This is confirmed by the presence of 100% homozygotes within the sub-population for IL-2. This population underwent non-random mating as a result of particular disruptive changes, thus a disturbance of the HWE. No genetic variability was witnessed for this locus within the sub-population (Keller and Waller 2002). However, the IFN-γ locus demonstrated an F_{IS} of -0.3333. Negative F_{IS} values represent excessive heterozygote presence due to outbreeding (Vanhala, Tuiskula-Haavisto et al. 1998), thus the case for the IFN-γ locus.

Table 4.9: Measure of heterozygote deficiency or excess in Victoria Market.

Allele/locus	**IL-2**	**IFN-γ**
A	1.0000	-0.3333
G	1.0000	-0.3333
Total	1.0000	-0.3333

The allele frequencies were calculated from the genotype frequencies, using the $p^2 + 2pq + q^2 = 1$ equation, where the AA genotype is p^2 and the GG genotype is q^2.

The Kwamashu and Victoria Market populations demonstrated significant chi square results ($p<0.05$) for the IL-2 locus. Significant chi-square values for the IL-2 locus have also been previously reported in broiler breeds (Kramer, Malek et al. 2003). However, the PMB population was the only one that showed an insignificant chi-square result ($p>0.05$). Hence, this confirms that there was no genetic variability in the PMB population as it deviated from the HWE (P=0.88, Table 4.10). Loss of genetic variability due to isolation and subdivision have also been reported for the IL-2 locus in Old Dutch breeds with no HWE (Kramer, Malek et al. 2003).

Observed homozygosity was greater than expected homozygosity for IFN-γ in the PMB and Kwamashu populations (Table 4.2 and 4.4). This was also the case for the IL-2 locus in Kwamashu and Victoria Market populations (Table 4.2 and 4.8). This may have affected interpretation of the results because overrepresentation of alleles as homozygotes can influence deviation from the HWE. Moreover, failure of a gel to separate 1bp variances between large fragments of IL-2 and IFN-γ may have caused some heterozygotes to be ignored. Hence, incorrect genotyping due to improper gel-making conditions may result in HWE deviation (Vanhala, Tuiskula-Haavisto et al. 1998). The Kwamashu and Victoria Market populations remained in HWE for the IL-2 locus (Table 4.10), but this was not the case for the PMB population. This indicates greater genetic variability for the Kwamashu and Victoria Market populations, and lower variability for PMB (table 4.10).

The most prevalent genotype was AA in all three populations for the IL-2 locus, but the Kwamashu population had the most prevalent AA genotype with a frequency of 0.56 (Table 4.10). Tohidi et al. (2012) also reported the AA genotype as the most prevalent in Malaysian RJF with a frequency of 0.93 for the IL-2 locus. The second most prevalent genotype was AG in all three populations, having the greatest prevalence coming from the PMB population with a frequency of 0.50. This was consistent with previous reports of Malaysian indigenous chickens, which reported the AG genotype in village breeds as the second most prevalent with a frequency of 0.46 (Tohidi, Idris et al. 2012). IL-2 had more heterozygotes in the PMB population compared to the Kwamashu and Victoria Market populations, and this can be explained by less genetic drift occurring in the PMB sub-population compared to those of the Kwamashu and Victoria market populations. Crossbreeding may have also occurred between chickens of the PMB population (Vanhala, Tuiskula-Haavisto et al. 1998). Then followed by the least prevalent genotype for the IL-2 locus, which was the GG genotype with a frequency of 0.15, and this was the case

for the current study (Table 4.10) with a frequency of 0.06. Although the GG genotype was the least prevalent, it has been reported to have a significant association with reduced SE loads in the spleen, caecum and liver of different chicken breeds (Tohidi, Idris et al. 2012) (Kramer, Malek et al. 2003).

Table 4.10: Genotypic and allelic frequencies of IL-2 in three different populations.

Populations	**Genotype Frequency**			**Allele Frequency**		**X^2**	**P**
	AA	AG	GG	A	G		
Pietermaritzburg Cedara	0.30	0.50	0.20	0.5476	0.4524	0.02	0.88
Kwamashu	0.56	0.38	0.06	0.7500	0.2500	6.72	0.00**
Victoria Market	0.44	0.44	0.11	0.667	0.3333	5.33	0.02**

Most populations for the IFN-γ locus showed statistically significant chi-square departure from HWE ($p<0.05$) except for the Victoria Market population ($p>0.05$, Table 4.11). For the IFN-γ locus, the GG genotype was the most prevalent in all three populations, and highest in the PMB population with a frequency of 0.77 (Table 4.11). The second most prevalent genotype was AA and AG genotypes of Kwamashu and Victoria Market populations with frequencies of 0.56. The least prevalent genotype was AA of the PMB population with a frequency of 0.02. Kramer, Malek et al. (2003) reported the non-significant effect of the IFN-γ genotypes GG and AA for bacterial loads in tissue organs. However, the AG mutation at 318bp of IFN-γ has been reported to have significant associations with priming primary Ab responses to *Brucella abortus* and SRBC. The synergistic interaction of IFN-γ by IL-2 on minimum secondary Ab titres (Y_{min}) to SRBC in F_2 offspring of M15.2 grandsires, and of the IFN-γ polymorphism on maximum secondary Ab titres (Y_{max}) of BA. Moreover, the interaction of IFN-γ by IgL on primary Ab response to SRBC and BA, and on time needed to achieve minimum secondary Ab titres (T_{min}) and Y_{min} of Ab response to SRBC in F_2 offspring of M5.1 grandsires was previously described (Zhou, Buitenhuis et al. 2001).

Table 4.11: The genotypic and allelic frequencies of IFN-γ in three different populations.

Populations	**Genotype Frequency**			**Allele Frequency**		**X^2**	**P**
	AA	AG	GG	A	G		
Pietermaritzburg Cedara	0.02	0.27	0.71	0.1579	0.8421	22.2	0.00**
Kwamashu	0.06	0.38	0.56	0.2500	0.7500	17.9	0.00**
Victoria Market	0.56	0.38	0.06	0.7500	0.2500	0.00	1.000

The overall mean of observed homozygosity and observed heterozygosity in Table 4.12 were calculated. The results showed that there was greater observed homozygosity (H_0=0.8100) than observed heterozygosity (H_e=0.1900) almost amongst all three populations. High homozygosity reflects high selection pressure (Johansson, Pettersson et al. 2010), whilst low levels of heterozygosity trigger the loss of alleles and this has been previously reported by Muir, Wong et al. (2008). Excessive homozygosity was a result of high levels of inbreeding amongst each population and extremely low levels of outbreeding amongst each population because of disruptive changes such as, non-random mating, natural selection, genetic drift, and genetic mutations. Thus, populations deviated from the HWE. The overall I value shows that 60.21% of genetic differentiation occurred among sub-populations and 39.79% occurred within sub-populations, and low genetic variability existed within the populations as a whole with migration limiting genetic divergence, thus keeping sub-populations similar.

Table 4.12 shows difference in allele and genotype frequencies for the IL-2 locus for all the three populations. This indicates an overall deficiency of heterozygotes in these populations due to lack of outbreeding and excessive levels of inbreeding; thus resulting into increased numbers of homozygotes. Therefore, there was very low genetic variability for all the three populations as an overall summary. Non-random mating caused the disturbance of the HWE by changing allele frequencies as a result of mutational changes within the populations, and inconsistent genotype frequencies due to natural selection

and genetic drift amongst sub-populations. Other reasons behind deviation from the HWE include, positive assortative mating that increases homozygosity, small population size that causes random sampling errors and unpredictable genotype frequencies (Dorak 2007).

Table 4.12: The overall mean values of each population genetic measure from three different populations of both the IL-2 and IFN-γ.

Population genetic measures	**Mean**
Shannon Fixation Index	0.6021
Observed homozygosity	0.8100
Observed heterozygosity	0.1900

The overall Fixation Index (F_{IT}) collaborates contributions from non-random mating within sub-populations (F_{IS}) and effects of random drift among sub-populations (F_{ST}). The overall F-statistics show that the extent of genetic inbreeding within sub-populations was 0.5256 and 0.5078 for the IL-2 and IFN-γ loci respectively (Table 4.13), however the IL-2 locus portrayed a greater F_{IS}, this is also verified in tables 4.5 and 4.8. A great amount of homozygotes was observed within populations and very low quantities of observed heterozygotes due to lack of outbreeding within the sub-populations for both loci. The overall F_{ST} for IL-2 and IFN-γ was 0.0305 and 0.2586, respectively. These F_{ST} values indicate that the extent of genetic differentiation amongst sub-populations was 0% for IL-2 and 29% for IFN-γ. Negligible genetic differentiation occurred for the IL-2 locus. However, very great genetic differentiation occurred for the IFN-γ locus, because differentiation greater that 0.25 is considered very great whilst that less than 0.05 is considered negligible (Weir and Cockerham 1984). However, the mean F_{ST} shows that there was 14% of genetic differentiation due to random drift amongst the sub-populations and 86% of genetic variation within sub-populations from non-random mating. Low genetic variability amongst these sub-populations reflects the occurrence of genetic drifts within sub-populations (Hartl, Clark et al. 1997).

Table 4.13: Overall summary of F-statistics and gene flow for all loci.

Locus	**F_{IS}**	**F_{IT}**	**F_{ST}**
IL-2	0.5256	0.5401	0.0305
IFN-γ	0.5078	0.6848	0.2856
Mean	0.5193	0.5865	0.1398

Chapter 5

General Conclusions and Recommendations

5.1 General Conclusions

Blood samples were successfully collected from Ovambo indigenous chickens from the PMB, Kwamashu and Victoria Market populations of Kwazulu-Natal, and genomic DNA was successfully extracted using the phenol-chloroform method. However, not all extracted DNA was in its pure form due to impurities such as those of water, protein inhibitors that may be present in buffers, and detergents. The quality of the extracted genomic DNA did affect the results of some downsream processes (e.g. amplification for the PCR product). Therefore, it is essential to demonstrate effective disruption of cells, denature nucleoprotein complexes, and inactivate nucleases such as DNase to prevent having contaminated genomic DNA.

The primer sets (forward and reverse) selected for the study effectively amplified the genomic DNA of the IL-2 and IFN-γ genes resulting into the expected PCR products. The PCR-RFLP assay fairly worked in genotyping for IL-2 and IFN-γ, demonstrating the expected fragments were observed. However, some fragments were not well separated due to carelessness with incubation temperatures, time taken to run the gels, high voltage, and incorrect percentage of the gel solution. Hence, considering these influencing factors can yield better results.

Population genetic measurements were determined using the Popgene software and proper analysis and interpretation of results was achieved. There was an overall of high levels of genetic differentiation amongst sub-populations compared to within sub-populations and excessive levels of homozygotes compared to inbreeding for both the IL-2 and IFN-γ loci. A substantial degree of heterozygotes was also observed for the IL-2 gene (PMB, figure) and IFN-γ (Victoria Market, figure). This indicated a high degree of non-random mating within the selected populations, due to disruptive changes such as, genetic drift, natural selection, gene flow, and changes of allele and genotype frequencies. However, genetic drift is the main source of limited divergence within sub-populations, thus low genetic variability. Most populations did not deviate from the HWE except for Victoria Market for the IFN-γ locus and PMB for the IL-2 locus. Deviation from HWE can be due to loss of genetic variability, isolation and subdivision within sub-populations and overrepresentation of alleles as homozygotes instead of heterozygotes. These population genetic measurements prove that when there is excessive inbreeding amongst indigenous chickens, genetic variability can be lost within a population.

It is of high importance to conserve indigenous chickens since they play an important part in the economy of both developed and undeveloped countries for the independence of small-holder farmers, and to maintain their genetic diversity. The weakness of the study was sampling from different areas of Kwazulu-Natal, which is just a portion of South Africa, thus sampling could have occurred in extremely different environmental backgrounds to prevent obtaining results of excessive inbreeding.

Detecting and genotyping for polymorphisms of the IL-2 and IFN-γ genes for disease resistance can further assist in the development of genetic lines through divergent genetic selection. These genetic lines can be crossbred to produce populations suitable for mapping molecular markers for desirable performance. The use of molecular markers can improve resistance to disease. Although, implementing genetic resistance is costly, it is the best approach for disease management as it can be a permanent solution. This will also help avoid the continuous use of vaccines and antibiotics on poultry, and reduce the cost and dangers of hazardous-disease challenge testing.

5.2 **Recommendations**

Although the PCR-RFLP method was used, microsatellites could have been used to analyze the genetic diversity of chickens, existing genetic relationships amongst and between populations, and for sub-division. Pyrosequencing could also be an additional tool that could have been used to produce genetic sequences of the gene that will help construct phylogenetic trees using the Nei's unbiased genetic distances to determine evolutionary relationships of the genes in different avian species. To prevent populations deviating from HWE and excessive inbreeding, a bigger sample size should be collected from totally different parts of the country selected to increase chances of genetic variation. The findings of this study show that IL-2 an IFN-γ are potential genes that can be adopted in selection programs for increasing disease resistance in indigenous chickens. However, these genes need to be exploited further before being utilized in selection programs. The inconsistencies of the results suggest that before genes are used in selection programs like marker-assisted selection, their markers should be nominated based on the basis of association scrutiny in the population in question. Progress of genetic resistant lines of South African indigenous chickens can also be a resolution after an in-depth analysis of the discussed genes. Genetic improvement in indigenous chickens is possible through the use of selection programs and cross-breeding, or combining the two for a more effective program. Although this would waste time it will be a permanent improvement. Breeds with better performance abilities can be used to improve the germplasm for cross-breeding for quicker genetic improvement (Padhi et al.2016). Proper management of

domesticated chickens with better food-feed and change in husbandry can improve their performance (Padhi et al.2016).

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