GENETIC DISTANCES OF TOLL-LIKE RECEPTOR 2 IN DIFFERENT BREEDS OF RABBIT

BY

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BENIN CITY, NIGERIA

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A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF ANIMAL SCIENCE, FACULTY OF AGRICULTURE, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF AGRICULTURE (B. AGRIC) DEGREE

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CERTIFICATION

This is to certify that this work was carried out by Miss Ogiesewu Nosawemwenze Esther with matriculation number AGR1600220 in the Department of Animal Science, Faculty of Agriculture, University of Benin, Benin City, Nigeria.

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Project Supervisor *Signature & Date*

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Head of Department *Signature & Date*

DEDICATION

This project is dedicated to God Almighty and to my Parents whose love and support kept me throughout my undergraduate years and during the course of this project.

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All thanks to God Almighty for his grace and mercy throughout this Project.

I want to sincerely thank my project supervisor, Prof. A. M. Orheruata for his guidance throughout this project, the time he spent going through my work and the corrections he made, his encouragement and fatherly support, may the good Lord continue to bless him. I am eternally grateful to Dr. B. Onaghise and Mr. Paul Aduba for their immense support and guidance towards the completion of this work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

ABSTRACT

Toll-like receptors (TLRs) initiate an innate immune response system (Chang, 2010). TLR2 genetic differentiation between populations could result from neutral processes or natural selection. The aim of this study was to determine the genetic distance of toll-like receptor 2 gene in breeds of rabbits. A total number of 40 adult rabbits (5months and above) were raised at the University of Benin teaching and research farm. Four (4) breeds of rabbits were selected for this experiment (Chinchilla, New Zealand, Hyla and Dutch breeds). About 2ml of blood was collected from the ear vein of the Rabbit separately and placed in a plain bottle containing 6ml of RNA shield. Blood samples were temporarily stored in a refrigerator before DNA extraction and analysis. Genomic DNA was isolated from each blood sample using the Zymo quick-g DNATM. Miniprep kit. Following the manufacturer's instructions. The results showed that there was no genetic diversity between Hyla male (F_1) x Chinchilla Male (Parent) as well as Hyla male (F_1 generation) x Hyla male (F_1). Furthermore, the degree to which Chinchilla Male (F_1generation) x Chinchilla Male (F_2generation), Chinchilla Male (Parent) x Chinchilla Male (F_1generation) and Hyla Female (Parent) x Chinchilla Male (F_1generation) differ was constant and the same, while the lowest degree of diversity in genetic Tol-like receptors was obtained from Chinchilla, Male (Parent) x Chinchilla, Male (Parent).

CHAPTER ONE

1.0 INTRODUCTION

Background to the Study

Rabbits provide an excellent source of animal protein for human consumption and may play a significant role in solving a part of meat shortage in Nigeria. Growth, the increase in live body mass or cell multiplication, is controlled genetically and environmentally.

Rabbit meat is rich in micronutrients such as iron, zinc, iodine, and vitamin B12 and low in cholesterol and sodium level (Adeolu *et al.,* 2020). Hence, its consumption was recommended for alleviating hidden hunger in women of reproductive age and infants within 1000 days (window period) of life. Despite some of the nutritional and health benefits of eating rabbit meat, rabbits exhibit exceptional phenotype diversity, which could serve great commercial benefits and also serve as important animal models in biomedical research (Carneiro *et al*., 2011). These attributes have brought about increased clamor and excitement for upgrading available rabbit breeds for high reproductive and growth potentials FAO, 2004).

rabbit has been used as an experimental animal in genetics and reproduction physiology since the beginning of the century, but it was not until 1950 that the

first findings on quantitative genetics were published, in Venge's study of maternal influence on rabbit birth weight (Venge, 1950). This work paved the way for research on the genetic improvement of the rabbit for meat production. Scientists at the National Institute for Agricultural Research (INRA) in France initiated research and development in this area in 1961, followed by work in other research laboratories in many countries, such as that of the teams of the University of Zagazig in Egypt, of Gödöllö and Kaposvar in Hungary, of Iztnanagar in India, of Milan and Viterbo in Italy, of Valencia, Saragossa and Barcelona in Spain, the Normal team in the United States and the Chinese teams (particularly in Shanghai) and those working in Nitra in Slovakia and in Cracow in Poland. Robinson's excellent 1958 bibliography in Genetic studies of the rabbit, based on sound genetic and physiological data, is now outdated by this new research. Work on rabbit genetics has been regularly updated at world rabbit congresses (Rouvier, 1980; Matheron and Poujardieu, 1984; Rochambeau, 1988).

However, experience gained under European production conditions cannot be transferred directly to developing countries. To upgrade their rabbits, breeders should use local animals, either native or from imported populations that have been locally adapted and make use of the genetic variability that is available. It does seem that priority should be given to research on rural and backyard rabbit production. These would be small, thrifty, autonomous units requiring little investment and using local resources. They would be reasonably productive.

Toll-like receptors (TLRs) are a major group of proteins that recognize molecular components of infectious agents, known as pathogen associated molecular patterns (PAMP). They are important mediators of the inflammatory response in the first line of host defense by recognition of many pathogen-related molecules and endogenous proteins associated with immune activation.

They are classified as members of the IL-1R super-family based on a shared cytoplasmic region known as the TIR (Toll/IL-1R) domain. TLRs are cell-surface receptors that induce a signal in the affected cell, involving a number of proteins, such as MyD88, IL-1 receptor-associated with extracellular leucinerich domains and an intracellular signaling domain and are found in monocytes, macrophages and neutrophils. TLRs have been implicated in the activation of macrophage by a variety of chemically diverse bacterial products (lipopolysaccharide (LPS), lipoproteins and peptidoglycans) and kinase and p38 mitogen-activated protein kinase (MAPK).

Among TLR genes, TLR2 is located on the outer membrane and forms a dimer complex with TLR1 or TLR6 to recognize peptidoglycans, lipoproteins or lipoteichoic acid of Gram-positive bacteria. This gene is widely expressed across species and recognizes the greatest number of PAMPs, detecting components from bacteria, viruses and fungi. TLR2 was suggested to exhibit high levels of polymorphism in several mammal species.

Toll-Like Receptors (TLR) is a receptor which plays role in innate immunity due to microbial infection. TLR is a membrane protein that helps receptor recognition pattern to various molecules derivatives from microbes and stimulate innate immunity due to microbe molecules exposure. TLR is known to be a recognition receptor which involves in pathogen associated molecular pattern (PAMP) recognized by pattern recognition molecules (PRMs) and a phagocytes development system in recognizing pathogen which can be stimulated any time to respond as inflammatory system. TLR stimulation through microbial product initiates signaling pathways which activates not only innate immunity but also adaptive immunity, and according to research result held by some researchers showed that TLR can also activate T-cells which play role in adaptive immunity. It has been known that TLR in lymphocytes act as essential signal to regulate lymphocytes activation and proliferation, produce antibody and regulate antigen presentation.

1.1 Justification

Toll-Like Receptors (TLR) is a receptor which plays role in innate immunity due to microbial infection. TLR is a membrane protein that helps receptor recognition pattern to various molecules derivatives from microbes and stimulate innate immunity due to microbe molecules exposure. Toll-like receptors (TLRs) are a major group of proteins that recognize molecular components of infectious agents,

known as pathogen associated molecular patterns (PAMP). They are important mediators of the inflammatory response in the first line of host defense by recognition of many pathogen-related molecules and endogenous proteins associated with immune activation. TLRs have been implicated in the activation of macrophage by a variety of chemically diverse bacterial products (lipopolysaccharide (LPS), lipoproteins, and peptidoglycans) and kinase and p38 mitogen-activated protein kinase (MAPK). TLR2 is located on the outer membrane and forms a dimer complex with TLR1 or TLR6 to recognize peptidoglycans, lipoproteins, or lipoteichoic acid of Gram-positive bacteria. Severla studies like Abrantes et al. (2013), Wang et al. (2994), Cargill et al. (2007) addressed different genetic diversity of the TLR3 in wild populations and domestic breeds, this study therefore seeks to investigate the genetic distances of TLR2 in different breeds of rabbit.

1.2 Objective

1. The broad objective of this study is the assessment of the Genetic diversity in different breeds of rabbits.

Specific objective

1. To determine the Genetic distance of toll-like receptor 2 gene in rabbit.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information on Rabbits

Domestic rabbits are the descendants of Oryctolagus cuniculus, a species native to the western Mediterranean basin (Spain and North Africa). Wild rabbits belong to other genera: Sylvilagus, Coprolagus, Nesolagus and Brachylagus. The rabbit was domesticated relatively recently: most breeds are created by humans and are no older than 200 or 300 years, which is why there are few locally adapted land races. Domestic rabbits are classified into different breeds based on the color, biometric traits, and origin (Sanford, 1996), but there is a need to differentiate between these rabbit breeds with the aid of molecular markers because they are more abundant, ubiquitous, hypervariable in nature, and evenly distributed along the chromosomes. Characterization at the molecular level is carried out to explore the genetic diversity between and within livestock populations and also to determine the genetic relationship among the different populations (Rahimi *et al*., 2015). Studies (Chantry-Darmon *et al*., 2006; Grimal *et al*., 2012; El-Aksher *et al*., 2016) have shown the importance of microsatellites in generating the information necessary for planning crossbreeding and selection of genotypes in genetic breeding programs in rabbits.

Rabbit meat is rich in micronutrients such as iron, zinc, iodine, and vitamin B12 and low in cholesterol and sodium level (Adeolu *et al*., 2020). Hence, its consumption was recommended for alleviating hidden hunger in women of reproductive age and infants within 1000 days (window period) of life. Despite some of the nutritional and health benefits of eating rabbit meat, rabbits exhibit exceptional phenotype diversity, which could serve great commercial benefits and also serve as important animal models in biomedical research (Carneiro *et al*., 2011). These attributes have brought about increased clamor and excitement for upgrading available rabbit breeds for high reproductive and growth potentials FAO, 2004).

2.2 Genetic distance of Rabbit

Genetic distance is a genetic divergence measurement between either species or populations within a species.

The adaptation of rabbit populations to climatic conditions may represent a genetic variability in their gene pool and this variability causes genetic differentiations among rabbit breeds/populations. The estimation of genetic variability of a species is an important criterion for its conservation and further genetic improvement. The genetic variability can be assessed by nuclear markers such as allozymes.. These are conservative genetic markers, evolving slowly but do not have much resolving power to reveal population differentiation. Other

molecular markers like AFLP (amplified fragment length polymorphism) have been used for several purposes like genetic analysis of inbred strains of rabbit (Oryctolagus cuniculas), for quantitative traits (Van Haeringen *et al*., 2001) and microsatellites for diversity analysis in wild rabbits (Zenger *et al*., 2003). random amplified polymorphic DNA (RAPD) technique is highly informative for understanding genetic relationship among the organisms, but it has low reproducibility (Williams *et al*., 1990). the RAPD-PCR has been used as a tool to assess the genetic variability and phylogenetic relationship among broiler rabbit breeds adapted in the semi-arid climate.

2.2.1 Genetic distances of Toll-like receptors

Toll-like receptors (TLRs) initiate an innate immune response system (Chang, 2010; Botos *et al*., 2011; Kawai and Akira, 2011; Kumar *et al*., 2009, 2011; Lee and Jin, 2008; Leulier and Lemaitre, 2008). They contain leucine rich repeat (LRR) domain in the ectodomain (ECD) and cytoplasmic signaling domains known as Toll IL-receptor (TIR) domains. The evolution of TLRs has been studied by many researchers. Phylogenetic analyses showed that the vertebrate TLRs evolved independently by gene duplication prior to the divergence of protostomes and deuterostomes (Hughes and Piontkivska, 2008; Roach *et al*., 2005).

TLR2 genetic differentiation between populations could result from neutral processes or natural selection.

2.2.2 Genetic diversity in rabbits

What is genetic diversity?

Diversity is another word for variation: the presence of differences among whatever you consider. Related to genetics, most obvious is the genetic diversity between populations. Genetic diversity represents the presence of genetic differences between animals within species, both between and within populations. Different breeds, for example, have specific genetically determined characteristics. Think about differences in size, colour, but also in purposes such as beef versus to dairy cattle or hunting versus guarding dogs.

Genetic diversity also exists within a population and is related to the genetic differences between animals in that population. It is possible, but very rare, that there is no genetic variation in a population. This occurs in populations that are fully inbred: animals are genetically completely identical to each other. But this is a very rare situation that may occur in genetic lines of laboratory animals that are especially created for that purpose. The purpose of those populations is to provide animals that are as genetically equal as possible so that genetic differences are not a cause of variation in, for example, testing new medicines. A population of clones would be even better from the point of view of having genetically equal animals. Such population would have no genetic variation at all.

2.2.3 Factors that affect genetic diversity

There are a number of forces that influence genetic diversity. Some can be influenced by us, others occur by coincidence. At population level there are forces that increase genetic diversity, and forces that decrease it. Mutations are events that create new alleles. When it happens in germ cells it has an increasing effect on genetic diversity. Also, migration may have an increasing effect, but only immigration (new animals moving into the population). Emigration (animals moving out of the population) usually has a decreasing effect on genetic diversity, especially when the population size is small. Also, selection has a decreasing effect: only animals with a specific genetic make-up are allowed to breed, at the expense of others.

2.3 Genetics of Rabbit Breeds and Population

A breed is a collection of individuals within a species which share a certain number of morphological and physiological characters which are passed on to their progeny as long as they breed among themselves.

One way of assessing the genetic uniqueness of different breeds is to study their origins. A breed is the outcome of the combined impact of artificial and natural selection (environmental adaptation). It is difficult to define exactly what is a breed and what is its background. Artificial selection may be based on a number of different criteria, not necessarily all to do with productivity. The breeding conditions may be either artificial or natural, the environment may gradually

change and so on. Rabbit breeds or populations can also be defined in terms of gene frequencies. This is possible with genes identifiable through their visible or major effects on progeny. Coloration and hair structure are classified as visible effects. Thanks to advanced observation techniques the genes governing blood groups, biochemical and protein polymorphism and hereditary are now also known. (See Zaragoza *et al*., 1990.) For quantitative characters, such as litter size or weight at weaning, which are controlled by a great many non-identifiable genes, rabbit populations can also be defined by their performance. These genes are also assumed to have little effect on overall variability and to function independently, according to the standard assumptions of quantitative genetics. Such characters are also influenced by the environment.

The environmental characteristics must be carefully described (number of breeders, the direction of selection, the origin of the population and its range) when describing a population. The genes are carried by chromosomes organized into 22 pairs (2n=44). About 60 markers have been described. These are genes of visible effect such as colour or coat or morphological anomalies, or genes coding for molecules of which the biological impact is being studied. These two approaches are hard to reconcile, for teams often use only one type of marker. Among the markers described, 37 have been placed on eight autosomes and on chromosome X; 23 markers constitute six linkage groups, and the locus of six

markers has still not been found. All these markers are spread over a majority of 22 pairs of rabbit chromosomes.

The links between the biological markers and the genes for colour or hair have rarely been tested, however. Experience has shown that the rabbit can support a slow and gradual increase in inbreeding, but research suggests that mating programmes for small populations should minimize its extent and rate of increase among the stock (Rochambeau, 1990). Breeds created by selectors, particularly amateurs in the United States and Europe, now conform to official standards. The book of the Fédération frawaise de cuniculture (FFC) on standards for rabbits describes more than 40 breeds. Each has been bred from animals of local and regional populations, or by crossing existing breeds, or by using mutants for changes in coat colour or structure. Mass selection for size and body morphology has separated these breeds into giant, medium, small and very small. It is interesting to study the origin of the breeds to learn whether they may correspond to original genetic ensembles and to attempt to determine their characteristics.

The characters by which an animal conforms to a breeding standard, such as body size, whether or not it is compact, coat colour and density and ear size, may be related to its resistance to variations in climate. In fact, such factors as coat, skin, body area and weight affect the animals' body temperature.

2.4 Toll-like Receptors

The Structure of Toll-like Receptors in Rabbits

TLRs were first found in drosophila and characterized by a transmembrane region followed by a cytoplasmic Toll/Interleukin-1 receptor (IL-1R) homology (TIR) domain and N-terminal leucine-rich repeats (LRRs) (Takeuchi and Akira, 2010), and they are vital for innate immunoresponse (Takeda *et al*., 2003). It has been known that the TLR family includes 13 genes in mammals, of which 10 have been identified in pig, cattle and human (Chang *et al*., 2006; Werling and Coffey, 2007; Chuang and Ulevitch, 2001; McGuire *et al*., 2006) and 12 in mouse (Oldenburg *et al*., 2012) and TLR1-like, 2, 3, 4, 5, 6, 8 and 10 in rabbit (Kajikawa *et al*., 2005; Astakhova *et al*., 2009; Abrantes *et al*., 2013).

Moreover, TLR2, 5, 6 and 10 were found on the membrane of cellular surface, while TLR3 and 8 are located in cytoplasm (Ohnishi *et al*., 2003; Schumann and Tapping, 2007). Various TLRs recognize the different PAMPs. Specifically, triacyl lipopeptides can be recognized by TLR1 or 2. Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphophatidyl inositol and mucin can be recognized by TLR2. Lipopolysaccharide from Gram-negative bacteria, mannan, glycoinositolphospholipids and envelop protein can be recognized by TLR4. Flagellin from bacteria can be recognized by TLR5. Diacyl lipopeptide, lipoteichoic acid and zymosan can be recognized by TLR2 or 6. The TLRs on cell

surface mainly recognize the component from bacteria, mycobacteria, candida, trypanosome, saccharomyces or streptococcus. Intracellular TLR3,7 and 8 can recognize ssRNA or dsRNA from virus and dsDNA from virus, CpG motifs from bacteria and plasmodium can be recognized by TLR9 (Tirumurugaan *et al*., 2010). Therefore, TLRs are vital for innate immunity.

TLRs are expressed predominantly in antigen processing and presentation cells such as macrophages, neutrophils and dendritic cells, but not restricted to these cell types. TLRs expressions have been detected in a wide range of tissue and their distribution of mammalian (Vahanan *et al*., 2008), and their expression levels have been associated with altered immune responsiveness (Menzies *et al*., 2006).

TLR2, 3, 4 and 9 are expressed differently in cervix, placenta and uterus of mice (Gonzalez *et al*., 2007). Lipopolysaccharide (LPS) a cell coat component of Gramnegative bacteria, can be recognized by TLR2 and 4. The opening reproductive system of female rabbit was easy to suffer from Gram-negative bacteria infection, such as Pasteurella, Brucella and Salmonella (Abrahams *et al*., 2004). Despite the crucial function of innate immunity mediate by TLR2 and 4 for host resisting Gram-negative bacteria, little known about the expression of TLR2 and 4 in different organs of female rabbit, and whether LPS affected the expression of TLR2 and TLR4 in genital tract of female rabbit.

2.5 Toll-like receptor 2

TLR2 is a major mammalian TLR that can recognize lipoproteins derived from bacteria, viruses, fungi, and parasites.TLR2 and TLR4 have been implicated in cellular responses to lipopolysaccharide (LPS), the major constituent of the Gram-negative bacterial outer membrane. TLR2 mediates cellular responses to structures from numerous microbial cell wall constituents and may thus be central in host recognition of diverse bacterial pathogens. SNPs in the TLR2 genes have been shown to be associated with suppressed macrophage response to mycobacteria in both animal and human studies.TLR2 knockout mice showed decreased resistance to tuberculosis (TB) on high-dose exposure.

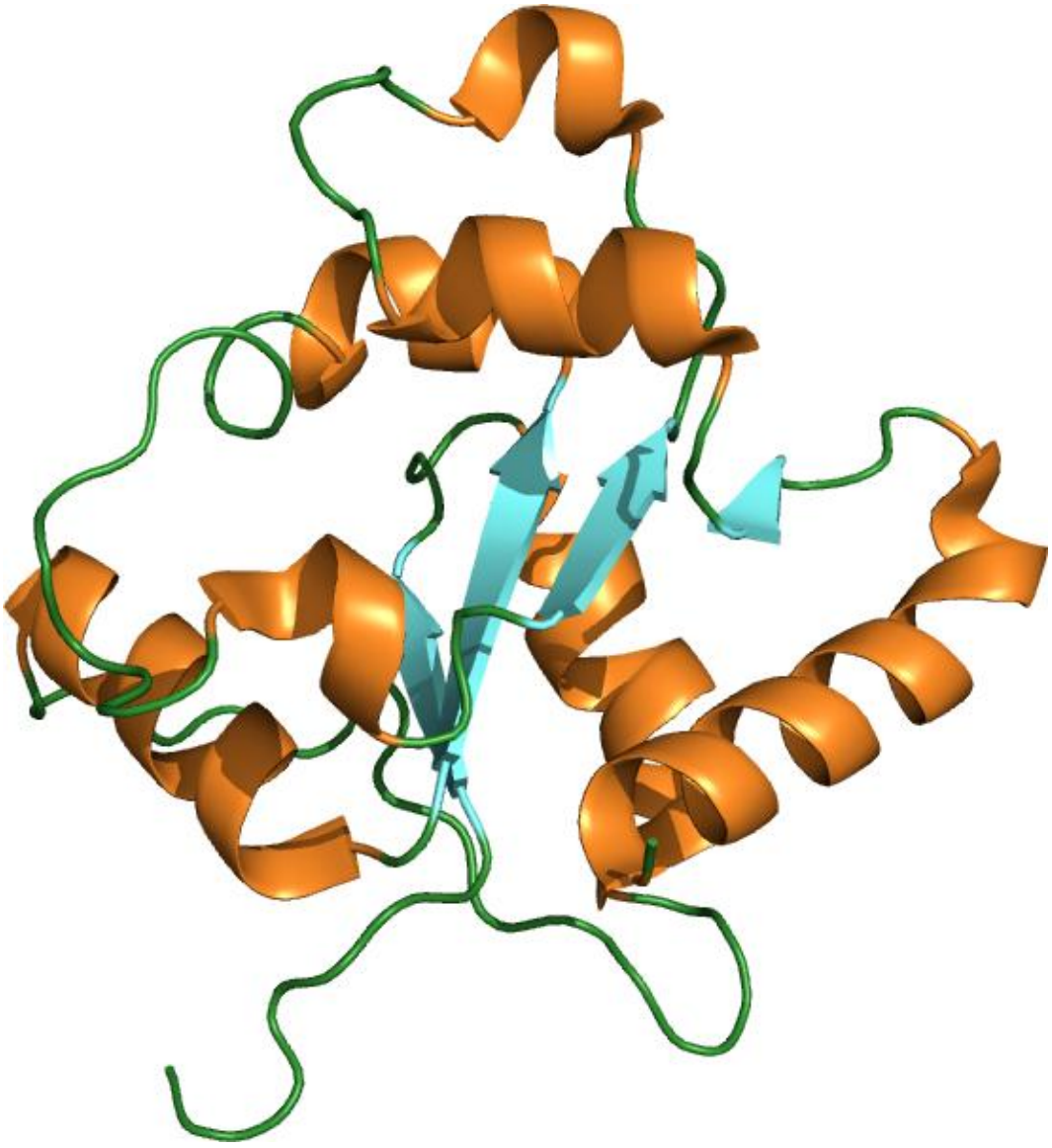

Plate 1: Structure of Toll-like receptor 2
Source: Saha (2015)

2.6 Mechanism of Toll-like Receptors

The immune system in mammals consists of innate and adaptive immune responses. Adaptive immunity is mediated by antigen specific T and B cells responses and is observed only in vertebrates. Innate immunity, however, is conserved between invertebrates and vertebrates. Toll-like Receptors (TLRs) play an important role in the recognition of components of pathogens and subsequent activation of the innate immune response, which then leads to development of adaptive immune responses. The TLRs are an ancient gene group which is found both in invertebrates and vertebrates; related genes are found also in plants. In mammals, members of the TLR gene family play a primary role in the recognition of pathogen-associated molecular patterns (PAMPs) in proteins from bacteria, viruses, protozoa and fungi. Mammalian TLRs derive their name from the Drosophila Toll protein, with which they share sequence similarity. The drosophila Toll protein was shown to be involved in dorsal-ventral pattern formation in fly embryos and was also implicated as a key component of host immunity against fungal infection.

The TLRs consist of a large extracellular domain responsible for PAMP binding, a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain which binds molecules and initiates cellular immune responses. The extracellular domains are composed of about 20 leucine-rich repeats (LRRs)

motifs of 20–30 amino acids (AA) and form a solenoid shape with the potential to bind the TLR specific PAMP.

Ten TLRs, which recognize molecular patterns from all major classes of pathogens, have been identified in mammals, eleven in mice. TLRs operate with diverse variety of ligands ranging from hydrophilic nucleic acid to LPS, furthermore the heterodimerization expands the ligand spectrum. TLR2 and TLR4 recognize bacterial cell components and are critical in the immune response against Gram positive and negative bacteria. TLR6 in association with TLR2 recognizes a wide variety of bacterial cell wall components including lipopolysaccharides, teichoic acid and lipoproteins and induce NFkB signaling pathway.

Several studies have shown that mutations in the TLR may reduce the ability of the protein to recognize PAMP and hence interfere with innate immune activation. Describing genetic variation in these loci in relation to resistance against specific diseases in livestock may be useful in guiding genetic selection for disease resistance. Single nucleotide polymorphisms (SNPs) within TLR genes in humans seem to be associated with susceptibility to infection by specific diseases. The initiation of the innate response to bovine respiratory syncytial virus (BRSV) requires the interaction of the viral F protein with TLR4, which leads to activation of NFkB via the Myd88-dependent pathway.

2.7 Rabbit Toll-Like Receptor and their Role in Pathogen Recognition

A major challenge to the application of selection for resistance to disease is the exposure to pathogens. Challenging breeding animals is generally impracticable and cannot guarantee the animals' welfare. Therefore, identification of DNA markers for disease resistance is widely studied and many useful single nucleotide polymorphisms (SNPs) are reported (Nicholas, 2005). Testing candidate genes is an approach used to search for DNA markers. In terms of innate immune system, pattern recognition receptors specifically recognize the molecular patterns derived from pathogens.

Toll-like receptors (*TLRs*) play important roles in recognizing "pathogen-associated molecular patterns (PAMPs)" of pathogens (such as viruses, bacteria and fungi; Abreu *et al.*, 2010). They may also initiate the early immune response in both innate and acquired immunity (Vasselon and Detmers, 2002). Mammalian *TLR* proteins contain an extracellular domain that consists of leucine-rich repeat (LRR) domains and an intracellular region that consists of a Toll/IL-1 receptor (*TIR/IL-1R*). The LRR domains are involved in ligand recognition and the *TIR/IL-1R* mediates signal transduction.

TLR2 recognizes PAMPs which are specific to microbes (Akira and Takeda, 2004). TLR2 could identify mycobacterial lipoglycan and other bacterial cell wall macroamphiphiles (*Ray et al., 2013*). As a result, it is reasonable to consider that *TLR2* is a critical candidate gene for studies of resistance or susceptibility to

bacterial infection in rabbits. It is well known that nucleotide change of the *TLR* genes may affect their ability to recognize PAMPs (Uenishi and Shinkai, 2009). Previous studies have demonstrated that the *TLR* genes could recognize slight differences among PAMPs (Janeway and Medzhitov, 2002). We can hypothesize that the sensitive recognition ability is due to polymorphisms in *TLRs*. Also, many studies of *TLR* genes have revealed an association between polymorphisms in the *TLR* genes and disease (Bochud *et al.*, 2007; He *et al.*, 2007; Abu-Amero *et al.,* 2013).

Polymorphisms in the human *TLR2* gene protect against malaria (Greene *et al.,* 2012). The Arg753Gln mutation in the human *TLR2* gene is associated with vitiligo susceptibility and urinary tract infection (Tabel *et al.,* 2007; Karaca *et al.*, 2013). A16934T polymorphism in the human *TLR2* gene is associated with severity of atopic dermatitis (Potaczek *et al.*, 2011).

2.8 Molecular structure of toll-like receptor

TLR ectodomains form a solenoid-shaped structure with the innerconcave surface comprised of b sheets with more unstructured loops forming the outer edge . TLRs bind to a number of diverse molecular structures, including lipids (e.g., TLR4: LPS via MD2; TLR2: lipoproteins), proteins (e.g.,TLR5: flagellin; TLR2 and TLR4: HMGB1), and nucleic acids (e.g., TLR3: dsRNA; TLR7/8: ssRNA; and TLR9: unmethylated CpG motifs in bacterial, viral, and fungal DNA) .

Several TLRs require cooperation with coreceptors for ligand binding, such as TLR1 or TLR6 for TLR2, MD2 for TLR4, and CD14 for TLR2, TLR4, and TLR3. The molecular interaction of ligands and TLRs could involve different molecular interfaces .For example, TLR3 binds dsRNA along the concave dimerization interface contacting 2 different sites on each monomer of the TLR3 dimer—one at the N-terminus and the other at the C-terminus of the solenoid. In contrast, both components of the TLR1/TLR2 heterodimer contribute to lipopeptide binding at the convex dimerization interface. For TLR5, there is a 2:2 conformation with a dimer of flagellin binding to a dimer of TLR5, but the flagellin binds on the lateral side of TLR5, not the side that forms the dimer interface . The ectodomain of membrane-associated TLRs is required for ligand binding and formation of functional dimers for signaling.

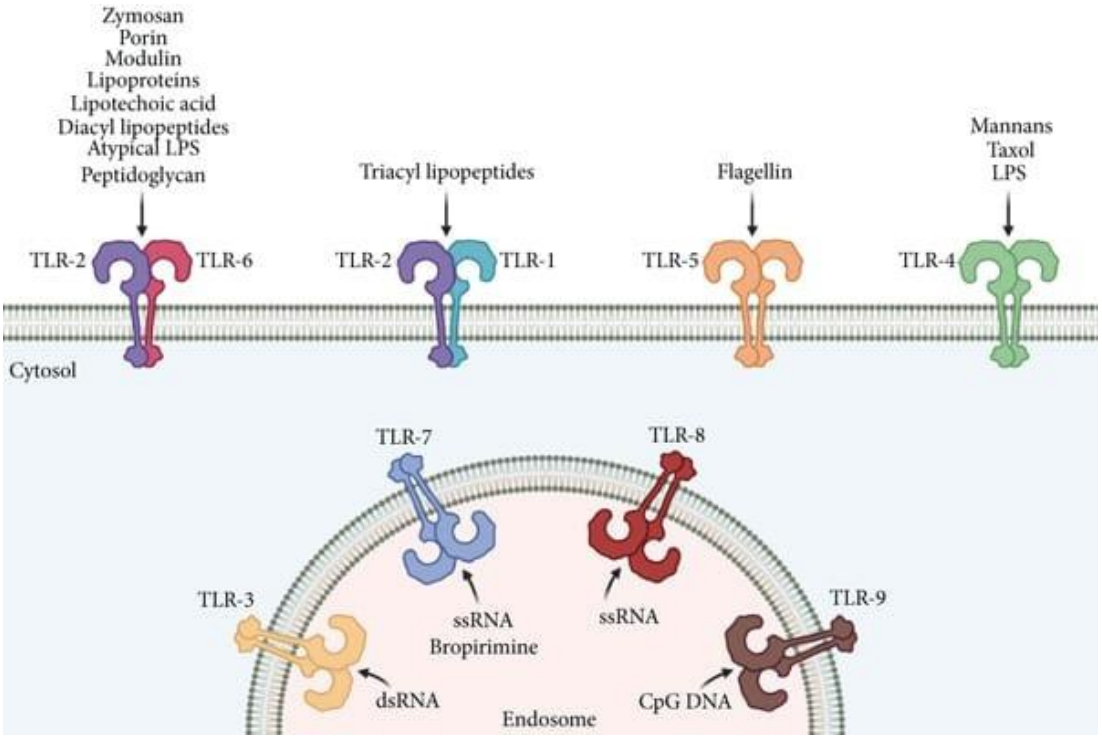

Plate 2: Molecular structure of Toll-like receptors
Source: Lucas et al. (2017)

CHAPTER THREE

MATERIALS AND METHOD

3.1 Experiment location

The experiment was conducted in the University of Benin teaching and research farm Edo state.it is located between latitude 6.3998^{o}N and longitude 5.6990^{o} E of the greenish meridian of the forest zone with a temperature of 28.6^{o}C ¨Annual rainfall ranges from 1498 to 3574 mm with mean value 2162 mm.the relative humidity ranges between 63.3% and 81.71% and daily sunshine between 5.8% and7.5 hours with mean value of 72.5 and 6.8% hours respectively(NAA,2015).

3.2 Experimental animal

A total number of 40 adults rabbits (5months and above) were raised in the university of Benin teaching and research farm. Four (4) breeds of rabbits were selected for this experiment (Chinchilla, New Zealand, Hyla and Dutch breeds).

3.3 Management

The rabbits were raised indoors in a wooden and metal hutch in the University of Benin Teaching and Research Farm.

experimental Materials used are steel hutch, metabolic feeders and drinkers, steel cages, feacal tray, sensitive scale and weigh balance. Compartment of hutches were constructed with steel material and wire mesh specially for housing the experimental animals. The cages were constructed in way that the feeders and drinkers are tightly fixed and detachable when necessary. Prior to Kindling the

entire hutches were repaired, cleaned as well as the equipment. The outside surrounding were also cleaned. Feeders and drinkers were specially designed to ensure zero wastage of feed and water. This enables taking account of remnant of feed per day.

A 5kg sensitive scale was used to measure the feed fed to the animals daily and their weekly body weight gain , birth weight, Average birth weight , weaning weight and Average weaning weight.

At the farm, mating were carried out at random within purebreds and also crossbred but always taking care to avoid full and half sibs as well as parent - offspring mating. The breeding buck was allowed to mate with three does. At the moment of parturition, the doe weight at kindling was measured, litters born were examined and the litter size at birth was recorded within 12h after kindling, and the litters were checked every morning to remove any dead kits. Weaning of kits took place on day 28 post - kindling and the litter size at weaning was recorded. All rabbits were kept in the same farm under the same environmental conditions throughout every stage of production. The rabbits were fed adlibitum on a pelleted commercial diet containing, around 16% crude protein and 14% crude fiber during the growing period from weaning till the starting point of production at 19 weeks of age and a diet containing around 18% crude protein, 12% crude fiber during production with a digestible energy of 2500 kcal/kg.

3.4 Blood sample collection

2ml of blood were collected from the ear vein of the Rabbit separately and placed in a plain bottle containing 6ml of RNA shield. Blood samples were temporarily stored in refrigerator before DNA extraction and analysis. Six (6) blood samples were collected from Chinchilla Male parent, F1 generation crossbred (CH × NZB) Male, Hyla female parent, F1 Hyla Male, F1 Dutch Male, and New Zealand black Male parent.

3.5 Laboratory analysis

Laboratory analysis were performed using laboratory facilities and equipment at Iqaba biotech laboratory Ibadan, Oyo state .

DNA extraction and protocol

Genomic DNA was isolated from each blood samples using the Zymo quick-$_g$ DNA^{TM}. Miniprep kit. Following the manufacturer's instructions. The kit a unique extraction technology for easy isolation of ultra-pure DNA from whole blood in less than 15 minutes, using clean spin-column technology.

Protocol

50 µl of blood from each samples was placed in a 1.5ml Eppendorf tube, and 200ul of Genomic Lysis Buffer was added .the mixture vortex for 5 seconds, and then allowed to stand at room temperature for 5 minutes .the Buffer RW2 was added to column and centrifuged at 11000* g for 2 minutes to dry membrane completely .the column was placed into a nuclease-free 1.5ml collection tube.

60 µl RNase-free water was added directly onto center of silica membrane and centrifuged at 11000 x g for 1 minute to elute RNA, which was immediately stored in refrigerator at -20^{o}C before further analysis.

Complementary DNA synthesis

Complementary DNA (cDNpA) was synthesized using SensiFAST™ cDNA synthesis kit (BIOLINE, USA), following manufacturer's instructions. The kit provides a rapid and very sensitive method for first strand cDNA synthesis for use in real-time PCR studies. All RNA samples were normalized to 0.57 ug for cDNA synthesis due to differences in the concentration of RNA samples. After normalization of RNA samples, 4 ul 5x TransAmp Buffer and I ul reverse transcriptase were added to each sample in a PCR tube and mixed gently by pipetting. The PCR conditions were 25°C for 10 minutes (primer annealing), 42°C for 15 minutes (reverse transcription), 48°C for 15 minutes (optional step). 85°C for 5 minutes (inactivation) and 4°C hold. After polymerase chain reaction, CDNA samples were stored at -20°C in refrigerator before real-time PCR.

Polymerase chain reaction (PCR) and Primers

After DNA extraction and quantification, three DNA samples from each genetics group were taken for TLR2 genes discovery and polymerase chain reaction (PCR). The primer used for PCR amplification of target gene (rabbitTLR2) were designed using the primer 3 and blast option at the NCBI database (www.ncbi.nlm.nih.gov) .The information on primers used is presented in table

1.Polymerase chain reactions (PCR) were perform in a 50µl reaction volume containing 10µl of 5X FIREPol® Master Mix(Solis BioDyne, Tartu, Estonia), 2.5µl each of forward and reverse primers,31µl of nuclease-free water and 4 µl sample DNA template. FIREPol Master Mix reagent composition include FIREPol DNA polymerase, 0.4 M Tris-HCL, 0.1 M (NH4)2SO4, 0.1%W/V Tween-20,12.5 Mm mgCL2, 1 Mm dNTPs (200µM each of dATP, dCTP, dGTP , dTTP), blue dye, yellow dye and compound that increases sample density for direct loading. PCR condition consist of 1 cycle of 95oc for 4 min initial denaturation , 35 cycles each of 95oC for 30 seconds denaturation, 62oC for 30 seconds annealing, 72oc for 1 min elongation , followed by 72oC for 10 minutes final elongation.

Table 3.1: Primers and sequences for Amplification of TLR2

Target molecules	Primer sequence	Product size (bp)	Annealing temperature (℃)
TLR2			
Sense	GGAAGCCTTTATGCCTTTGC	683	62.67
Antisense	TTCTCGCAGGCTGAATTTTT		

Preparation of Agarose Gel

1.5% (w/v) agarose gel was prepared by adding 0.3 g of agarose powder into a flat-bottomed conical flask containing 20 ml of 1 X TBE (Tris Borate EDTA) buffer. The mixture was placed in a microwave for about 2 minutes to dissolve the agarose powder. The solution was cooled by running water on the side of the

flask. 1 µl of ethidium bromide was added to the solution and swirled gently. The solution was poured into a casting tray with sealed edges and a sample comb was slotted vertically into the solution to create wells in the gel before it solidifies. The solution was allowed to solidify horizontally for about 20 minutes. forward and reverse primers, 4 µl of template cDNA and 14.8 µl of nuclease-free water in a smart cycler tube. A 25 µl non-template reaction mixture was also run alongside negative control. The qPCR conditions were as follows: 40 cycles each of initial denaturation at 95°C for 15 minutes; denaturation at 95°C for 15 s; annealing at 65°C for 20 s and extension at 72°C for 20 s.

Electrophoresis of PCR products

After polymerase chain reaction (PCR), 1.5% agarose gel was removed from casting tray and submerged in electrophoresis tank. 1X TBE (Tris Borate EDTA) was poured into the electrophoresis tank. 5 ul each of PCR products were carefully loaded separately into wells on the agarose gel. A molecular-weight size marker (Hyper Ladder 100 bp. BIOLINE, USA) was also loaded into a separate well alongside DNA samples in order to estimate the size of the amplified DNA fragment. The estimation of the size of amplified fragments is important in order to confirm the amplification of the right DNA fragment (rTLR2 gene). After loading samples into the gel, electrophoresis was done at 240 A and 100 V for 20 minutes.

Visualization of PCR products (amplicons)

After agarose gel electrophoresis, the agarose gel was carefully placed in a trans-illuminator and viewed under ultraviolet (UV) light. The presence of bands (approximately 764 bp) is a confirmation that the right gene target has been amplified, and photograph of gel was taken.

Cleaning of amplicons

Amplicons were cleaned using Zymo DNA Clean and ConcentratorTM -5 (Zymo Research Corporation, USA) following the manufacturer's instructions.

Protocol:

210 µl of DNA loading buffer was added to 42 ul of amplicon (PCR product) in a 1.5 ml micro-centrifuge tube and vortexed briefly. The mixture was transferred to a Zymo-Spin Column in a collection tube. The mixture was centrifuged for 30 seconds, and then the flow-through was discarded. 200 µl of DNA Wash Buffer was added to the Spin-Column and centrifuged for 30 seconds, and the wash step was repeated. 30 µl of DNA Elution Buffer was added directly to the Spin-Column matric and incubated at room temperature for 1 minute. The Spin-Column was transferred to a 1.5 ml micro-centrifuge tube and centrifuged for 30 seconds to elute the DNA.

Agarose Gel Electrophoresis of cleaned amplicons

4 µl of each amplicon was mixed with 1 µl of DNA loading dye in PCR tubes, by pipetting up and down. Each sample was loaded into separate well in a 1.5 %

agarose gel. 5 ul of molecular-weight size marker, 100 bp. was also loaded in a separate well as a standard to estimate the size of the amplicons. Electrophoresis of samples was performed at 240 A and 100 V for 20 minutes. After electrophoresis, the gel was carefully transferred to a trans-illuminator (BIOLOGIX) and viewed under ultraviolet (UV) light to verify the presence of amplified fragments of rTLR2 gene. The presence of bands (approximately 764 bp) confirmed the amplification of the target rTLR2 gene.

Sequencing of PCR products

A quality check of PCR products of rTLR2 gene was performed by agarose-gel electrophoresis. The DNA sequencing of rTLR2 gene was done with the same PCR primers .using the Sanger Sequencing Chemistry, and Q20 read length up to 800 bases with ABI file were reported.

Alignment and Editing of sequences

Sequences of rTLR2 gene from the different breeds of rabbits (Chinchilla, New Zealand, Dutch, hyla) were aligned and edited using Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0.

mixture was transferred to a spin-column in a collection tube and centrifuged at 10000 X for 1 minute. The collection tube was discarded with the flow through. The spin-column was then transferred to a new collection tube. 200 ul of DNA Pre-wash Buffer was added to the spin-column and centrifuged at 10000 X g for 1 minute. Then, 500 ul of g-DNA Wash Buffer was added to the spin-column and

centrifuged at 10000 X g for 1 minute. The spin-column was transferred to a clean 1.5 ml micro-centrifuge tube. 50 µl of DNA Elation Buffer was added to the spin-column and allowed to incubate at room temperature for 5 minutes. The tube was centrifuged at top speed (16000 X g) for 30 seconds to elute the DNA, which was stored at-20°C for downstream analysis.

DNA quantification and integrity

The concentration and purity of each DNA sample was determined using Nano drop Spectrophotometer. 2µl of each DNA sample was used to determined DNA purity and concentration. DNA sample with 260/230 ratio of 1.5 to 2.5 were used for further analysis.

Genetic distance estimation

The estimation of pairwise genetic distance of TLR2gene between the different breeds of rabbits (Chinchilla, New Zealand, Dutch, Hyla) collected was performed using the p-disance method (Nei and Kumar, 2000) as implemented in MEGA 7.0 software (Kumar *et al*, 2016).

CHAPTER FOUR

RESULTS

Table 4.1 shows the degree to which the different species are distant in terms of genetic makeup

	1		2		3		4
1	0.1489473746	**%**					
2	0.1489473746	**%**	0.0046606217	**%**		**%**	
3	0.1603440218	**%**	0.0000000000	**%**	0.0000000000	**%**	
4	0.1489473746	**%**	0.0000000000	**%**	0.0000000000	**%**	0.0000000000

1 = Chinchilla, Male (F_1 generation); 2 = Chinchilla, Male (Parent); 3 = Hyla, Male (F_1 generation); 4 = Hyla, Female (Parent).

CHAPTER FIVE

DISCUSSION

The results from table 4.1 show that there is no genetic diversity between Hyla male(F_1) x Chinchilla Male(Parent) as well as Hyla male(F_1 generation) x Hyla male(F_1). This could be due to the fact that they are both males (Gunia *et al*. 2015). Rabbits' resistance to pathogenic diseases can be fairly ascertained using visual inspection. Rabbits exhibit exceptional phenotypic diversity, are of great commercial value, and serve as important animal models in biomedical research (Carneiro *et al.* 2011). The work of Carneiro *et al.* 2011 indicates that most of the total genetic variation is within breeds as opposed to between breeds.

The degree to which Chinchilla Male (F_1generation) x Chinchilla Male (F_1generation), Chinchilla Male (Parent) x Chinchilla Male (F_1generation) and Hyla Female (Parent) x Chinchilla Male (F_1generation) differ is constant and the same. The reason for this might be that Chinchilla Male (F_1generation) and Chinchilla Male (Parent), although are of same species, they belong to different filial generations. This could also imply that Chinchilla Male (Parent) and Hyla Female (Parent) have similar degree of resistance to pathogens as the Toll-like receptor are genetically similar. This is similar to the work of Gunia *et al*. 2018, who suggested that genotype by environment interactions played a major role in affecting the disease resistance offered by any particular rabbit species. Toll-like

receptors (TLRs) serve a key role in modulating immunological responses to a variety of pathogen-derived ligands and linking adaptive immunity with innate immunity. TLRs are key mediators of inflammatory pathways in the gut. Domestic rabbits frequently contract pasteurellosis, a bacterial infection brought on by *Pasteurella multocida.* It is very contagious and mainly spreads through direct touch, though it can also spread through coughing or sneezing. In colonies of rabbits, between 30% and 90% of rabbits that appear healthy could actually be disease-free carriers. The symptoms of pasteurellosis include rhinitis (runny nose), pneumonia, abscesses (sores packed with pus), infections of the reproductive system, head tilt, and blood infections literature suggests that naturally breeding species with resistant genes(TLRs) in a healthy environment will help to reduce the effect and spread of the disease .

Toll-like receptors (TLRs) are evolutionarily conserved receptors belonging to the family of pattern recognition receptors (PRRs) which play a vital role in immune responses especially pathogen recognition by the extracellular matrix (Akira *et al.,* 2001; Takeuchi & Akira, 2010; Takeda & Akira, 2015). TLRs are directly involved in the regulation of inflammatory reactions and activation of the innate or adaptive immune responses for the elimination of infectious pathogens (Delneste, 2007, Jing *et al.,*2012) TLRs hold a key position in the first line of defense against pathogens because of their ability to recognize the conserved pathogen-associated molecular patterns (PAMPs), conserved structures of the

pathogens, or the damage caused by the pathogens within the host (Kang and Lee 2011; Behzadi *et al*., 2021) Pattern recognition receptors (PRRs) make up a key component of the innate immunity because of their ability to initially sense the exposure to infection and elicit an intracellular signaling cascade for the eventual elimination of the pathogen and infected cell thereof (Takeuchi & Akira, 2010). All PRRs are germline-encoded proteins which can recognize wide varieties of alien molecules (e.g., lipid, carbohydrate, peptide, and nucleic acid) commonly found in pathogens but distinct from the host molecules and thus are commonly referred to as PAMPs (Lee *et al*., 2012) The highest degree of diversity of in genetic characters was found in Hyla, Male (F_1 generation) x Chinchilla, Male (F_1 generation). This suggests that Chinchilla species have the highest resistance to pathogens with respect to the other species used in the experiment (Nowland *et al.*, 2015). This resistance could be attributed to the Toll-like receptor present in their cell wall.

But the lowest degree of diversity in genetic is obtained from Chinchilla, Male (Parent) x Chinchilla, Male (Parent). This result may be due to the fact that the species are the same. Dale, (2021) suggests that the most obvious effects of inbreeding are poorer reproductive efficiency including higher mortality rates, lower growth rates and a higher frequency of hereditary abnormalities. This has been shown by numerous studies with cattle, horses, sheep, swine and laboratory animals. Wang *et al.*,(2020) believes that A strain shall be regarded as inbred

when it has been mated brother × sister or offspring × parent, also inbreeding has a negative effect on the economic traits and immune response.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

According to the experiment's findings, among the species included in the study, chinchilla species had the most advanced toll-like receptors and the greatest genetic diversity. Therefore, it is advised that rabbit farmers raise chinchilla species if they want to breed rabbits that are resistant to pathogens. To clarify the precise evolutionary relationships between the species studied in the study, more research is required.

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