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Genetic Selection Pressure on the Toll-like Receptor 9 Gene (*TLR9-rs187084)* Increase Individual Susceptibility to Systemic Inflammatory Disease: A Case Study of the Kano State Population

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Abstract:
Toll-like receptor 9 (*TLR9*) may recognize and generate an immunological response to DNA double-strand; therefore, its link with experimental and serological lupus symptoms must be investigated. Genetic data on the normal frequencies of the *TLR9* (rs187084) polymorphism may make it difficult to choose an adequate sample size for case-control association studies. This study investigated how *TLR9* (rs187084) polymorphism is typically distributed among the people living in Kano, Nigerians. *TLR9* (rs187084) polymorphism was examined in a sample of 315 individuals using the polylmerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The mutant allele was more common (67%) and gender-independent. The gradual incidence of changed alleles in both identities suggests the population was chosen to survive tuberculosis (TB) and malaria. Though increased *TLR9* expression is protective against infections, it might likewise play a part in the development of inflammatory disorders. The high frequency of *TLR9* (rs187084) over-expression in Kano State residents may be the result of genetic adaptation to the state's common infectious and parasitic diseases. This is thought to be the outcome of interactions among genetic factors and the terrain. Systemic lupus erythematosus (SLE) and other autoimmune disorders have been linked to this genetic plasticity. Sample sizes for future case-control studies can be determined using the current data. However, to further understand the link between the TLR9-rs187084 variation and the risk of systemic inflammatory diseases, functional investigations might be undertaken.

Keywords: *TLR9 Gene; Inflammatory disease; Polymorphism, Population; PCR-RFLP.*

1. Introduction

Toll-like receptors, often known as TLRs, are the first line of defense in the body's defense against microbes [1]. They recognize the disease-causing agent as well as endogenous danger indicators that are present in dead cells and wounded tissues, which links innate immunity with adaptive immunity. TLRs are found in a broad variety of cells, including immune cells and other cells [2]. The effects of TLR signaling on cells vary widely depending on the receptor, the kind of cell, and the co-stimulatory signals present. TLRs are responsible for the production of cytokines, which stimulate inflammation, cell formation, and survival, as well as communication between the innate and adaptive immune systems [3]. It has been shown that humans have a total of 10 TLRs (TLR1–10), mice have a total of 13 TLRs (*TLR1–13*), and *Drosophila melanogaster* has a total of 9 TLRs [4, 5]. The duties that belong to the family of human TLRs are carried out by means of a convoluted signaling cascade that, in the end, has an effect on the production of a broad variety of inflammatory cytokines [6]. TLRs are essential components of the first immune response to infections because of their ability to recognize conserved pathogen-associated molecular patterns (PAMPs), conserved pathogen structures, or host damage generated by disease-causing agents [7]. This ability makes TLRs important players in the fight against infectious diseases. In addition to immune response cells, TLRs have been identified in a wide range of different cell types, including macrophages, neutrophils, dendritic cells, natural killer cells, mast cells, basophils, and eosinophils [4]. The *TLR9* gene is on the short arm of chromosome 3 (3p21.2), and its codes for a protein with 1032 amino acid residues and a molecular weight of 115.8

kDa [6, 8]. *TLR9* is a unique receptor that can recognize unmethylated DNA variants from viral and bacterial pathogens [9]. *TLR9* has been discovered to include two single nucleotide polymorphisms (SNPs), rs187084 and rs352140. These SNPs have recently been associated with inflammatory disorders such as diabetes mellitus, lupus, SLE, asthma, osteoarthritis, and rheumatoid arthritis [6, 8, 10]. There are now known to be ten functional *TLRs*, named *TLR1–TLR10*, expressed by immune and certain non-immune cells in humans. There are 10 different *TLRs*, with *TLR1*, 2, 4, 5, 6, and 10 all being surface proteins and *TLR3*, 7, 8, and 9 all being endosomal or endocytic proteins. Changes in the innate immune system that are specific to the host, like the *TLR9* gene, can change the course of an adaptive immune response. This is especially true if an infection lasts for a long time. Polymorphisms in the *TLR9* gene have been identified and linked to a variety of illnesses, including bacterial meningitis, cytomegalovirus infection, toxoplasmosis, malaria, and systemic lupus erythematosus.

However, most research has focused on the *TLR9* 1635A/G polymorphism (rs352140), which has been linked to an increased risk of various different types of infectious illness. The 1635A>G polymorphism in *TLR9* has also been looked at in relation to HIV infection, and it has been shown to be linked to HIV transmission, disease progression, CD4 cell counts, and viral load. There may be genetic variances between the research populations that account for the different study results. However, how this polymorphism really affects HIV disease characteristics is still a mystery. The *TLR9* polymorphism 1486C/T (rs187084) in the promoter region has been linked to SLE, rheumatoid arthritis, HPV infection, pulmonary tuberculosis, and some types of cancer [4, 8, 11].

It has been reported that *TLR9* variants contribute to the development of asthma, SLE, IBD, and atherosclerosis. *TLR9* single nucleotide polymorphisms (SNPs) have been linked to a variety of complications. Specifically, SNP G2848A (which is equivalent to T−1237C) is associated with myocardial infarction, deep venous thrombosis, and chronic obstructive pulmonary disease (COPD). Limited statistical significance has been reported for the contribution of the C allele of T−1237C to an elevated susceptibility to asthma among European Americans [11]. However, a cohort study of Japanese individuals revealed that this factor had no impact on the occurrence of asthma [12]. In two more studies, there was no link between *TLR9* single nucleotide polymorphisms (SNPs) and asthma or coronary artery re-stenosis after percutaneous coronary intervention [13, 14]. In a separate investigation, the promoter SNP T−1486C and the intron 1 variant G1174A of *TLR9* were analysed in 440 patients with SLE in comparison to 406 controls. With marginal statistical significance, the 1174G allele was more prevalent in lupus patients (51.6% of cases versus 44.0% of controls; P = 0.0291). A frequent co-inherited variant of this allele was allele −1486C. Functional analysis of the GC haplotype indicated that, in comparison to the ancestral AT haplotype, this variant decreased *TLR9* transcription [15]. An association between *TLR9* variants and susceptibility to lupus in Korea, China, and the United Kingdom, respectively, was not established in three additional studies [16, 17]. The *TLR9* gene is located near a susceptibility locus for Crohn's disease and ulcerative colitis, prompting multiple authors to investigate probable links between *TLR9* polymorphisms and IBD [18]. Research revealed that the presence of −1237C was linked to the development of Crohn's disease, but not ulcerative colitis, in a cohort of individuals from Germany [19]. An investigation conducted on Italian patients with inflammatory bowel disease (IBD) found that the *TLR9* −1237C variant was much more prevalent in those experiencing frequent bouts of pouchitis (45.7%) compared to those with fewer occurrences (20.9%) [20]. A DNA study looked at the link between *TLR9* polymorphisms and the risk of developing primary biliary cirrhosis (PBC). The study found no link between *TLR9* SNPs and the risk of PBC [21].

Systemic inflammatory diseases, such as sepsis, inflammatory bowel disease (IBD), and rheumatoid arthritis (RA), pose substantial health issues worldwide. Discovering the genetic characteristics that make people more susceptible to certain disorders has the potential to improve the evaluation of risk, enable early identification, and facilitate the development of personalized treatment approaches. The proposed study aims to identify new genetic connections with systemic inflammatory disease in the

Kano State population. The primary goal of this study was to investigate the prevalence of the *TLR9* (rs187084) polymorphism among the Kano State population in Nigeria. This would contribute to a better understanding of how these diseases develop.

2. Materials and Methods

2.1 Study Population

The current study was conducted inside Kano State's populace over the course of one year, beginning on August 9th, 2021, and winding down on August 8th, 2022. Three hundred and fifteen (315) individuals from Kano State, Nigeria, who seemed to be in good health, volunteered to take part in the study. We excluded participants with a current or past medical history of diabetes mellitus, hypertension, cancer, or an autoimmune condition in their family. A written permission form, which included an explanation of the goal of the study, was provided to each participant before they were allowed to take part in the research.

2.2 Blood Sample Collection

From 315 study population peripheral venous blood sample (5ml) was collected in a sterile tube containing EDTA as an anticoagulant. The blood sample collected were properly levelled and stored at -20°C until further analyses.

2.3 Isolation of Genomic DNA and PCR Amplification

Using a Bio-Rad extraction kit and according to the instructions supplied by the manufacturer, genomic DNA was extracted from 5 ml of intravenous blood drawn from each participant. In each PCR reaction, which was carried out in a volume of 25 µl, 1 µl of genomic DNA, 1 µl of forward and reverse primers, 3µl of standard PCR buffer, 1 µl of dNTPS, 0.2 µl of Taq DNA polymerase, and 17.8µl of water were used. The reactions were carried out in a total volume of 25 µl. The reaction was carried out using a PCR machine from Bio-Rad with the following parameters: initial denaturation at 95°c for five minutes, followed by thirty cycles of 95°c for thirty seconds, 59°c for thirty seconds, and 72°c for thirty seconds of annealing and extension, respectively. The temperature was 72°c during the last ten minutes of the extension. Prior to being analyzed, the PCR item was kept at a temperature of 4°C.

2.4 TLR 9 (rs187084) Genotyping

Screening for the TLR9 (rs187084) variant was performed using PCR-RFLP in line with the approach that had been reported before [6]. The PCR-RFLP analysis was carried out in a reaction volume of 20 µl, which included 7.75 µl of water, 2 µl of buffer, 0.25 µl of Afl II enzyme, and 10 µl of PCR product. Following an overnight incubation of the reaction mixture at 37°C, the processed samples were separated by electrophoresis on 2% agarose gel, and the results were seen under ultraviolet light. Confirmation of the genotyping was achieved by the use of nucleotide sequencing (Macrogen, Korea).

2.5 Statistical Analysis

The direct gene counting approach was used for the purpose of determining the genome and allele frequency rates. The X2 method was used in order to make the determination of whether or not the observed frequencies were in a state of Hardy-Weinberg equilibrium. The statistical package SPSS, version 17.0 (IBM, United States), was used for the aim of data analysis. If the p value was lower than 0.05, then the hypothesis was regarded as having statistically significant support.

3. Results and Discussion

3.1 Demographic Details of The Participants Involved In The Study

The study group consisted of 165 males, with a normal age of 43.74± 17.24 years, and 150 females, with a regular age of 39.65± 17.83 years. All the participants have signed an informed writing consent

form. The participants' ages ranged from 42 to 17 and half years old on average. **Table 1** presents the findings for the remaining demographic characteristics. It was determined that the observed genotype frequency among the people who participated in our research agreed with the Hardy-Weinberg equilibrium.

Table 1: presents the age range characteristics of the participants involved in the study.

Demographic details	Total	Males (n = 162)	Females (n = 153)
Male Age (years)	41.08	42.82	37.85
Standard deviation (years)	17.67	17.43	17.65
Standard error of mean (years)	1.165	1.417	1.784
Median Age (years)	44	44	44

3.2 Primers and Restriction Enzyme Used in The Study

Table 2 provides a description of the primers used, together with the corresponding annealing temperature and enzyme employed, within the scope of this research endeavor. The *TLR9*-1486T/C (rs187084) polymorphism was identified by the use of the restriction enzyme Afl-II on a 565-bp PCR-amplified fragment.

Table 2: Primer and restriction enzyme used for TLR9 (rs187084) genotyping

SNP ID	Primers	AT	SIZE	Restriction enzyme
TLR9 (rs187084)	F: TTCATTCATTCAGCCTTCACTCA	59°C	565bp	Afl-II
	R: GAGTCAAAGCCACAGTCCACA			

AT= Annealing temperature, bp= base pair, Afl-II= Restriction enzyme, TLR= Toll-like receptor

3.3 Polymerase Chain Reaction Restriction Fragment Length Polymorphism Results

The results of our analysis are shown in Table 3, which details the frequency distribution of the ancestral and changed alleles of the *TLR9* (rs187084) variation among the people who took part in our study. A similar trend was also detected in the frequency of mutant alleles among men (66%), females (63%), and individuals who had been stratified according to their gender. Males exhibited a somewhat greater occurrence of mutant alleles compared to females.

Table 3: Frequencies of genotypes and alleles for the TLR9 (rs187084) polymorphism

TLR 9 (rs187084) Genotype	Genotype frequency (%) in Kano State Nigerian (n =315)
TT	160 (51%)
CT	105 (33%)
CC (ancestral)	50 (16%)
Allele	Frequency (%)
C (ancestral)	205 (33%)
T (mutant allele)	425 (67%)

TT= Mutant allele, CC= Ancestral allele, % = Percentage, TLR= Toll-like receptor

The intracellular and ligand-binding domains of TLRs are particularly susceptible to structural alteration in response to polymorphisms and mutations in the TLR genes. The TIR domain may have an impact on the occurrence, severity, and prognosis of numerous immune-related disorders [22]. Research suggests that the TIR domain may have a role in the development, severity, and overall outcome of a variety of immune-related diseases. Numerous studies have produced data that demonstrates the presence of a wide variety of genetic variations that have an effect on the vast majority of genes encoding Toll-like receptors (TLR). When the host is exposed to dangerous diseases,

these polymorphisms and the associated pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) can affect how the immune system responds [23-25]. A change in only one nucleotide in a gene may have repercussions for the structure and function of the encoded protein [8]. Numerous single nucleotide polymorphisms (SNPs) included within the TLR gene, in particular those situated in the N-terminal region and leucine-rich repeat motifs, have been shown to have a major impact on the ability of the immune system to recognize pathogens [22]. Both rs187084 and rs352140 are examples of SNPs related to *TLR9*, and recent research has shown that both of these SNPs are associated with inflammatory diseases [8]. This is the very first study that we are aware of that investigates the frequency of the *TLR9* (rs187084) variation in the population of Kano State, which is situated in the country of Nigeria.

We discover that the incidence of the minor allele (T) in Kano State (67.0%) was consistent with previous results (65.0%) from the Tamil community [26] and (64.0%) from the North Indian population [27]. This was the finding that we made after doing our research. There is a correlation between the frequency of mutant alleles found in Iranians (62%), Chinese (63%), and Koreans (65%) when compared to the rates reported for other groups [28-30]. In spite of this, it was found that the prevalence of the minor allele was 53% in Japanese [31]. The frequency of the mutant allele is lower in Caucasian groups (55–59%) as compared to the population in Kano State, Nigeria [30].

The mutant allele "T" is associated with an increase in *TLR 9* expression in cells, as shown by the findings of functional study [31]. Carriers of the mutant allele had a lower risk of contracting diseases including TB and malaria, according to research that was carried out on people from India and Africa. It was discovered that the mutant allele was connected to the decreased parasite burden in Africans, although the connection was not statistically significant [28]. Because the mutant allele T protects against the most prevalent endemic illnesses, the findings of these studies show that the *TLR9* gene has been exposed to the pressures of genetic selection in order to withstand endemic diseases [32]. We are able to corroborate, based on our prior findings in the Tamil community as well as our present findings in the Nigerian group, that high-risk populations have genetic alterations that enable them to cope with illnesses that are frequent in their region. These findings come from both of our research groups. After separating the subjects into male and female groups, the researchers discovered that the mutant allele was present in equal numbers in both groups. This suggests that comparable experiences with environmental risks and chances for genetic adaptation were shared by these populations.

In populations where the *TLR9* (rs187084) mutant allele "T" is more common, which may be viewed as a genetic variation to control widespread infections, the risk of developing autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Inflammatory Bowel Disease (IBD) may be higher than in other populations [33, 34]. Animal models of autoimmune illnesses such as SLE have verified the function that *TLR9* plays in the development of disease owing to its capacity to recognize nucleic acids and stimulate the immune system [27]. The *TLR9*-1486 C/T (rs187084) polymorphism was discovered to dramatically enhance the risk of developing SLE in the Han Chinese population [33]. Researchers who were looking for genetic risk factors for SLE [34] found that the *TLR9*-1486C/T (rs187084) polymorphism had an effect on the production of autoantibodies in a South Indian population.

Another *TLR9* mutant allele (rs5743836) has been connected to an enhanced danger of developing SLE, and this trend has also been observed among South Indian Tamils [35]. Overexpression of the *TLR9* protein, which might enhance SLE pathophysiology via triggering and intensifying the autoimmune replies counter to the nuclear mechanisms [36] via the TLR/type I Interferon pathway [35], a severe variant of SLE has been associated with Africans and Indians. The participation of TLRs in the pathogenesis of SLE and the likely therapeutic goals related to TLRs for the treatment of SLE has been examined in previous studies. [37]. The SLE is caused by complex interactions between genes and the environment. To learn more about how these interactions lead to lupus, screenings will need to be done to find more variants in *TLR9* and other genetic risk factors**.**

4. Conclusion

The results of this research reveal novel insights into the ways in which the *TLR9* gene interacts with the genetic composition of the population of Kano State. People living in Kano State had a greater incidence of the *TLR9* (rs187084) over-expression mutation than the average population. There is a possibility that this is the result of a shift in genetics or the pressure exerted by natural selection to be able to combat prevalent endemic infectious and parasitic diseases. On the other hand, genetic adaptability puts carriers at a higher risk of developing autoimmune illnesses such as SLE and others. This is because of the complex interactions that occur between genes and the settings in which they are found. The frequency distribution of the *TLR9* (rs187084) variant that was discovered in these most recent results may now be used to calculate the appropriate sample size for any future case-control research. The fact that the findings of our research may not be applicable to populations located outside of Kano State due to genetic, environmental, lifestyle, and other types of variable variances is one of the limitations of our research. Future case control studies might utilize the normative frequency of the *TLR9* (rs187084) polymorphism found in our cohort to determine the appropriate sample size. Also, functional investigations might be carried out to explain the molecular processes behind the observed link between the *TLR9*-rs187084 variation and systemic inflammatory disease susceptibility.

Authors' Contribution

Yusuf Jibril Habib: Conceptualization, Conducted the research, assessed the results and wrote the original draft. Mohammed Sani Jaafaru: Review and editing, and visualization of the manuscript.

Data Availability

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare there are no competing interests.

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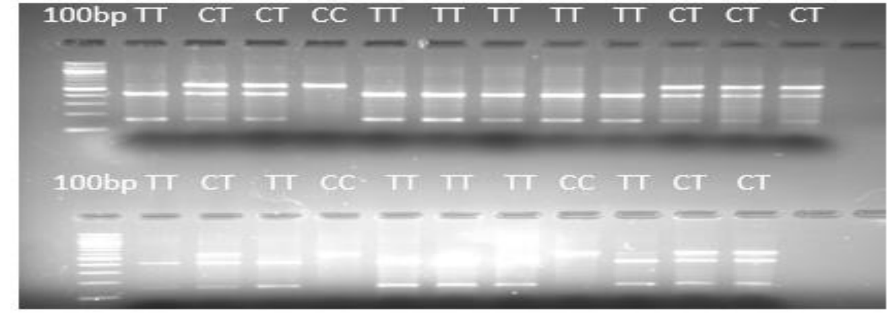
Appendices:

1-RFLP Gel Picture 10-9-2021
23 samples



TT=12 (415+149), CT=8 (565+416+149), CC= 3 (565 No RE Site)

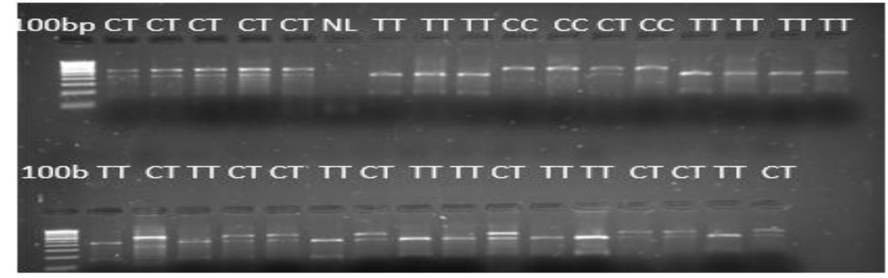
2-RFLP Gel Picture 20-9-2021
17 samples

Figure 13: Agarose gel electrophoresis of digested product.
J129, J130, J131, J136, J137, J138, J139 TT (416+149)
J123, J124, J125, J126, 127, J134 CT (565+416+149)
J132, J133, J135 CC (565)

3-RFLP Gel Picture 25-9-2021
16 samples

Figure 14: Agarose gel electrophoresis of digested product.
J140, J142, J145, J147, J148, J150, J151, J154 TT (416+149)
J141, J143, JI44, J146, J149, J152, J153, J155 CT (565+416+149)

4. RFLP-Gel Picture 28-10-2021
33 Samples



TT= 15 (416+149), CT= 14 (565+416+149), CC=3 (565 No RE Site), NL=1

5-RFLP Gel Picture 30-10-2021
16 samples

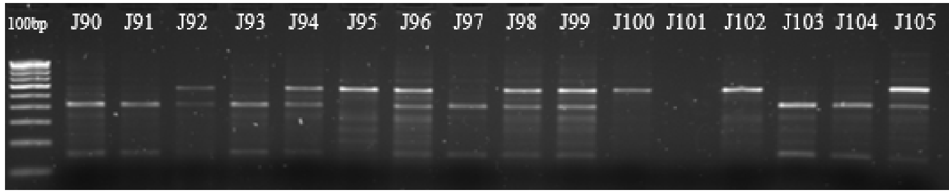


Figure 11: Agarose gel electrophoresis of digested product.
J90, J91, J93, J97, J103, J104 TT (416+149)
J92, J94, J96, J98, J99, J105 CT (565+416+149)
J95, J100, J102 CC (565)

6-RFLP Gel Picture 3-01-2022
17 samples

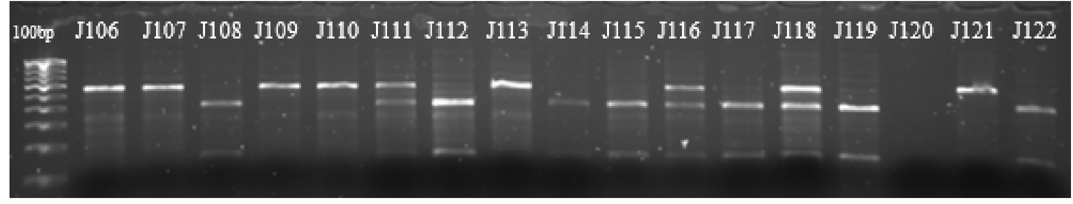
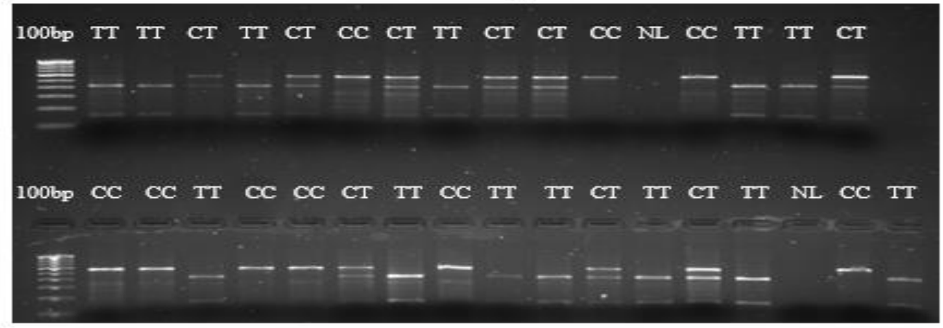


Figure 12: Agarose gel electrophoresis of digested product.
J108,J112,J114, J115, J117, J119, J122 TT (416+149)
J111, J116, J118 CT (565+416+149
J106, J107, J109, J110, J113, J121 CC (565)

7-RFLP Gel Picture 29-01-2022 33 Sample



TT= 13 (416+149), CT= 9 (565+416+149) CC= 9 (565 No RE Site) NL=2

8-RFLP Gel Picture 3-2-2022 16 samples

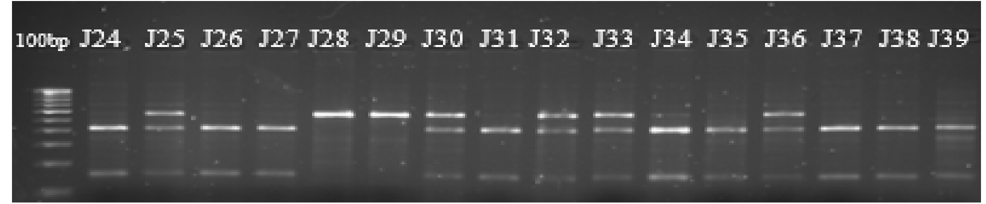


Figure 7: Agarose gel electrophoresis of digested product.
J24, J26, J27, J31, J34, J35, J37, J38, J39 TT (416+149)
J25, J30, J32, J33, J36 CT (565+416+149)
J28, J29 CC (565)

9-RFLP Gel Picture 20-2-2022 17 samples

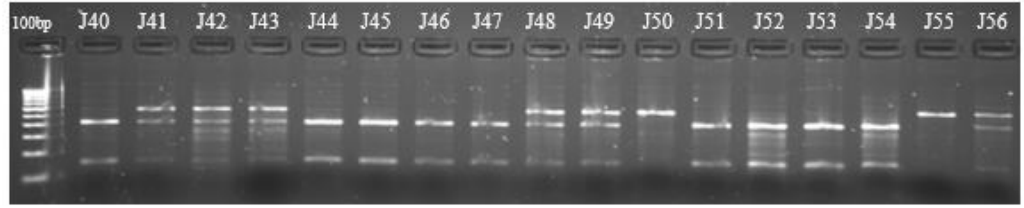


Figure 8: Agarose gel electrophoresis of digested product.
J40, J44, J45, J46, J47, J51, J52, J53, J54 TT (416+149)
J41, J42, J43, J48, J49, J56 CT (565+416+149)
J50, J55 CC (565)

10-RFLP Gel Picture 12-3-2022
16 samples

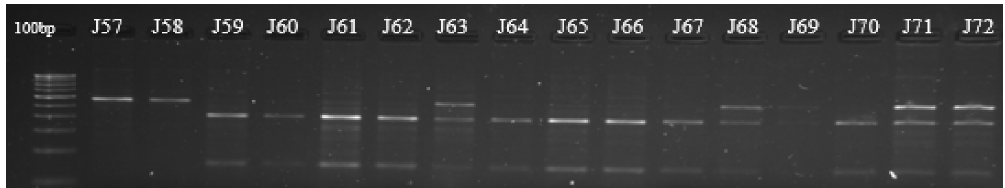


Figure 9: Agarose gel electrophoresis of digested product..
J59, J60, J61, J62, J64, J65, J66, J67,J70 TT (416+149)
J63, J68, J71, J72 CT (565+416+149)
J57, J58, CC (565)

11-RFLP Gel Picture 23-3-2022
16 samples

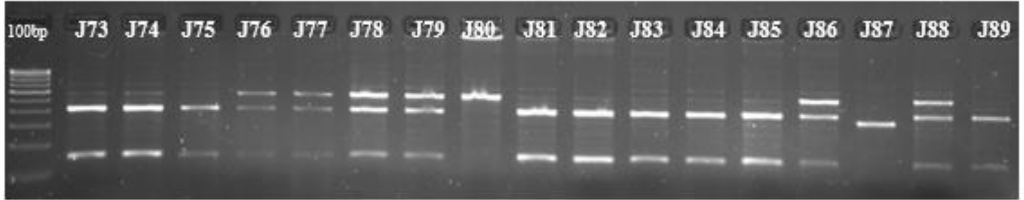
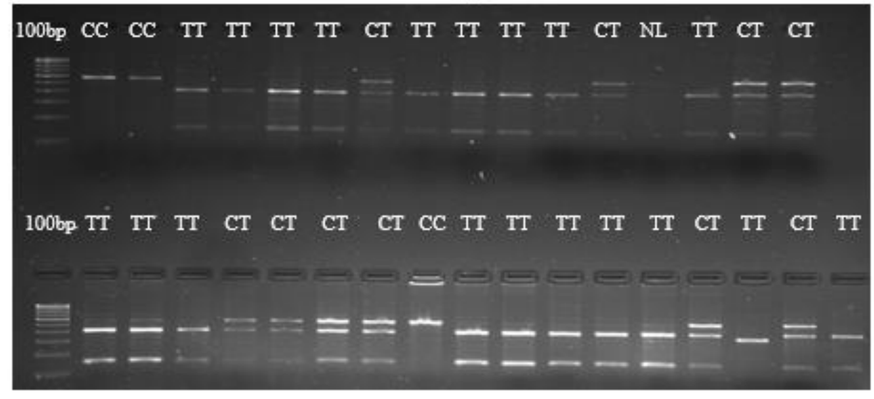


Figure 10: Agarose gel electrophoresis of digested product.
J73, J74, J75, J81, J82, J83, J84, J85, J87, J89 TT (416+149)
J76, J77, J78, J79, J86, J88 CT (565+416+149)
J80 CC (565)

12-RFLP Gel Picture 27-3-2022
33 Samples



TT= 19 (416+149), CT= 10 (565+416+149), CC= 3 (565 No RE Site) NL=1

13-RFLP Gel Picture 2-4-2022
13 Samples

Figure 15: Agarose gel electrophoresis of digested product.
J165, J171 **TT (416+149)**
J159, J160, J161, J162, J163, J164, J167, J168, J169, J170 **CT(565+416+149)**
J166 **CC (565)**

14-RFLP Gel Picture 5-4-2022
13 Samples

Figure 16: Agarose gel electrophoresis of digested product
J174, J175, J177, J178, J179, J181, J184, J185, J187, J188 **TT (416+149)**
J173, J180, J183 **CT (565+416+149)**
J172, J176 **CC (565)**

15-RFLP Gel Picture 15-4-2022
16 Samples

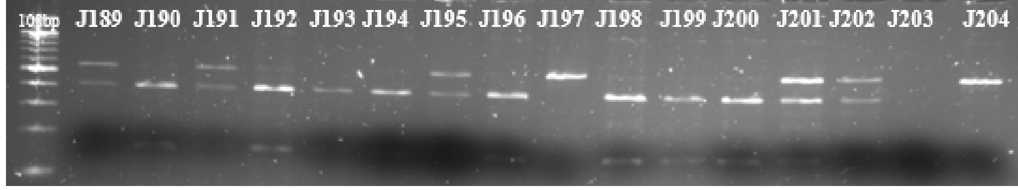


Figure 17: Agarose gel electrophoresis of digested product
J190, J192, J193, J194, J196, J198, J199, J200 **TT (416+149)**
J189, J191, J195, J201, JJ202 **CT (565+416+149)**
J197, J204 **CC (565)**

16-RFLP Gel Picture 20-4-2022
17 Samples

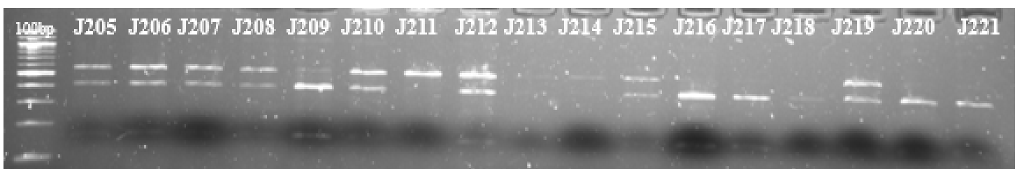
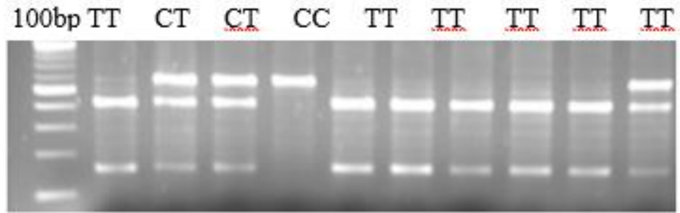


Figure 18: Agarose gel electrophoresis of digested product.

J209, JJ216, J217, J218, J220, J221	**TT (416+149)**
J205, J206, J207, J208, J210, J112, J215, J219	**CT(565+416+149)**
J211	**CC (565)**

17-RFLP Gel Picture 25-4-2022
10 Samples



TT= 6 (415+149) CT= 3 (565+416+149) CC= 1 (565 No RE Site)