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Haptoglobin Gene Polymorphism and Association with Microalbumiuria among Type 2 Diabetic Patients With and without Microalbuminuria in Kano State, Nigeria

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Abstract

Background: Diabetic nephropathy (DN) is one of the major complications that increase steeply along with diabetes epidemic. Microalbuminuria (MA) serve in the early detection of DN. Haptoglobin gene is polymorphic with 3 phenotypes (Hp1-1, Hp2-1 and Hp 2-2) and susceptibility to DN development is haptoglobin phenotype type dependent. The aim of the study was to evaluate the association of Hp gene polymorphism and MA in type 2 diabetic patient attending some selected hospital in Kano State, Nigeria.

Method: The study recruited 84 normotensive type 2 diabetic patients with and without MA and 84 age and sex matched apparently healthy individuals. Plasma glucose, urea, creatinine, Glycated haemoglobin (HbA1c), urinary creatinine, urinary albumin and microalbuinuria were analysed from the samples. Polymerase chain reaction was done to determne the haptoglobin phenotypes. Statistical anaylysis was done using SPSS version 25.0.

Result: Comparison of anthropometric and biochemical parameter between the Haptoglobin phenotype groups shows no statistical significance except for ACR. A prevalence of 20.2%, 34.6% and 45.2% were observed for the Hp1-1 Hp2-1 and Hp2-2 respectively. Hp1-1 and Hp2-1 were found not to be risk factors for the development of MA among diabetic patients. However, Hp2-2 was found to be a risk factor for the development of MA among diabetic patients.

Conclusion: The study found that Hp1–1, Hp2–1, and Hp2–2 haptoglobin genotypes are present among patients with type 2 diabetes mellitus. Hp1–1 and Hp2–1 were not associated with microalbuminuria, whereas the Hp2–2 genotype was significantly associated with an increased risk of microalbuminuria, indicating its potential role as a genetic risk factor for early diabetic nephropathy.

INTRODUCTION

The origin and etiology of DM can vary greatly but always include defects in either insulin secretion or response or in both at some point in the course of the disease[1]. Diabetic nephropathy (DN), is defined as albuminuria (albumin excretion rate > 300 mg/24 h) with a decline in renal function in a patient with known diabetes and in the absence of urinary tract infection or any other renal disease. Diabetic nephropathy is one of the major complications of diabetes mellitus of public importance. Early detection of diabetic nephropathy is therefore important in order to prevent or slow the progression of patients to end stage renal disease. For the early detection of diabetic nephropathy, biomarkers predictive of the onset of DN play an important role and the best-known biomarker for DN is microalbuminuria testing.

Microalbuminuria (MA) is an early marker of nephropathy[2]. Therefore, early detection and appropriate interventions in asymptomatic individuals may help in preventing deterioration in renal function and progression to diabetic nephropathy and end stage renal disease[3]. Also, in determining why some diabetic patients develop these complications while others do not; genetic differences between diabetic patients might play an important role.

Haptoglobin is an acute plasma protein that is synthesized mainly in the liver[4,5]. It is characterized by its strong affinity to bind free haemoglobin after haemolysis. The Hp gene is located on chromosomes 16q22.3 in humans[6,4]. The haptoglobin locus is polymorphic with two codominant alleles (Hp1 and Hp2)[6]. In humans, there is a common polymorphism of the haptoglobin gene characterized by alleles Hp 1 and Hp 2. The principal difference between alleles Hp 1 and Hp 2 is the presence of a duplicated DNA segment of 1700 bp in Hp 2 but not Hp 1[7]. There are three major phenotypes at the protein level: Hp1-1. Hp 2-1 and Hp 2-2 are inherited in a Mendelian pattern[4].

The functional properties of haptoglobin are type dependent as it is well established. Hp 1-1 is a better antioxidant and binds more strongly with free hemoglobin than Hp 2-2. The increased antioxidant function of Hp 1-1 is thought to confer protection from angiopathies, conversely, Hp 2-2 is believed to be a major risk factor in several oxidative stress-related disease states. Hp2-2 exists as large circular polymers, having decreased binding affinity for free hemoglobin, and has been associated with biomarkers of oxidant stress and iron delocalization[8]. It is well proven that the functional properties of Hp are type dependent. Hp 1-1 is a better antioxidant and binds more strongly with free hemoglobin than Hp 2-2[9].

MATERIALS AND METHODS

Eighty-four Type 2 diabetic patients (18-80 years) with (ACR of 30-300 mg/g) and without microalbuminuria (ACR of less than 30 mg/g) attending Aminu Kano Teaching Hospital, Kano, Murtala Muhammad Specialist Hospital and Muhammad Abdullahi Wase Specialist Hospital were included in this study consecutively. The control group were eighty-four (84) (age and sex matched) apparently healthy individuals from the blood donor clinic. Patients excluded include hypertensive type 2 diabetic patients, type 2 diabetic patients with other complications likely to affect the renal integrity like smoking, fever, vigorous exercise, urinary tract infection, and drugs. Patients with diabetic macroalbuminuria and cardiovascular disease were also excluded. Fasting blood sample (10 hours) was collected in ethylene diamine tetra acetic acid EDTA (for glycated haemoglobin and DNA analysis), fluoride oxalate (for glucose) and plain (for urea and creatinine) containers. Urine samples were collected randomly in sterile container and used for urinalysis and microalbumin analysis. All pre-analytical errors were avoided, and proper sample transportation was ensured.

An ethical approval was obtained from the ethical committee of Aminu Kano Teaching Hospital Kano before the commencement of sampling. Likewise ethical approval was collected from Health and Research Ethical Committee of Kano State Ministry of Health. Selected patients were duly informed about the research and consent form signed willingly. A structured interviewer questionnaire was administered to obtain necessary information on the subject's blood pressure, age and anthropometric variables such as height, weight and history of DM.

Molecular Analysis

DNA Extraction

Genomic DNA extraction was done using the QIAamp Blood Mini kit and according to the manufacturer's instruction (Qiagen). Into 1.5 ml microcentrifuge tube, 20 µl Protease was dispensed. Sample of 200 µl was added. Addition of 200 µl of Buffer AL was followed by pulse- vortex mixing for 15 seconds. It was incubated at 56^{o}C for 10 minutes. The 1.5 ml microcentrifuge tube was centrifuged briefly to remove drops from the lid. Then, 200 µl of ethanol (96-100%) was added. Pulse- vortex mixing for 15 seconds and brief centrifugation of the tube to remove drops from the lid was done. The mixture was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim and the cap closed. This was centrifuged at 6000 □ g (8000 rpm) for 1 minute. The QIAamp mini spin column was placed in a clean 2 ml collection tube while the tube containing the filtrate discarded.

The QIAamp Mini spin column was carefully opened and addition of 500 µl of Buffer AW1 followed without wetting the rim. The cap was closed and centrifuged at 6000 □ g (8000 rpm) for 1minutes.The QIAamp Mini spin column was placed in a clean 2 ml collection tubewhile the tube containing the filtrate discarded. The QIAamp Mini spin column wascarefully opened and 500 µl of Buffer AW2 was added without wetting the rim The cap was closed and centrifuged at full speed 20000 □ g (14000 rpm) for 3 minutes. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and tube containing the filtrate discarded This step was repeated. The QIAamp Mini spin column was placed on a new spin column and was briefly centrifuged. 50 µl of elusion buffer was added and allowed to stand at room temperature for 10 minutes. It was centrifuged at full speed (20000 □ g for 5 minutes. The QIAamp Mini spin column containing the eluted DNA was removed and stored at -20 ^{o}C and the other column discarded.

Primer Design

The Hp 1 gene sequence was adopted from the studies of Koch *et al*.[7]. The specificities of selected template were checked using nucleotide blast. Table 3.1 shows the selected template.

Primer set used for Hp 1 and Hp2 genotyping

Oligonucleotide primers 5'-GAGGGGAGCTTGCCTTTCCATTG-3' and 5'- GAGATTTTTGAGCCCTGGCTGGT-3' were used for amplification of a 1757-bp Hp 1 allele-specific sequence and a 3481-bp Hp 2 allele-specific sequence.Primers 5'-CCTGCCTCGTATTAACTGCACCAT-3' and 5'-CCGAGTGCTCCACATAGCCATGT-3' were used to amplify a 349-bp Hp 2 allele-specific sequence.

DNA Amplification using PCR

PCR machine manufactured by Genetouch Company, China with brand name Genetouchthermocycler and model TC-E-48FA was used. Koch *et al.*[7]protocol using reagent manufactured by Qiagen was used and all the manufacturer's instructions were followed. Each PCR mix of 20 µl contained 2 U of Taq polymerase, 1-100 ng of DNA, 200 µM each of dNTP, 0.15 µM each of forward and reverse primer and 1 □

PCR buffer.The following PCR conditions were used: initial denaturation at 95^{o}C for 5 minutes, followed by 35 cycles at 94^{o}C for 45 seconds,60^{o}C for 1 minute, 72^{o}C for 1minute,final extension at 72^{o}C for 10 minutes, then held at 4^{o}C

PCR Optimisation

Amplification procedure was aimed at amplifying at 1757 base pair fragment of Hp1, 3481 base pair Hp2 and 349 base pairfragment of Hp2. To achieve this, several trials proved abortive because there were no specific amplification using the company provided annealing temperature. To obtain the optimal annealing temperature, gradient PCR was adopted. A gradient of 50 ^{o}C to 60^{o}C was set using the gradient function blockand this was possible by setting 10 ^{o}C gradient for the primer. The set used is as followed: denaturation at 94 ^{o}C for 45 seconds, annealing at 50^{o}C to 60^{o}C for 1 minute and extension at 72 ^{o}C for 1 minute. Well temperature of 55^{o}C was found to be the best condition.

Visualisation of PCR Product

PCR products were separated by loading in 2% agarose gel electrophoresis for 20 minutes at 10 V/cm. The gel was stained with ethidium bromide during preparation. The gel was then viewed using the gel documentation system where bands were seen. Gel documentation system manufactured by Syngene Company, UK with brand name Ingenius3 and model IG3/1459 was used.

STATISTICAL ANALYSIS

The results were expressed as mean ± SD. Statistical analysis was performed using statistical package for social science (SPSS) version 25.0 (SPSS Inc. Chicago, IL, U.S.A.). One-way analysis of variance was used to assess the comparison between the parameters of the Hp phenotypes. Chi square was used to check for relationship. Multiple regression analysis was used to check for relationship. Odd ratio was also used to check for risk. Significant level was set at $p<0.05$. Significant level was set at $p<0.05$.

RESULT

Table 1 presented the mean values of creatinine, urea, ACR, HbA1c, DDT, FBG and ACR among the haptoglobin phenotypes. No statistical significance was found in creatinine (p=0.199), urea(p=0.303), FBG(p=0.923), HbA1c(p=0.687), BMI(p=0.184) and DD(p=0.538) among the Hp phenotypes. However, a statistical significance was found when ACR (p=0.007) was compared among the Hp phenotypes. ANOVA was used to obtain the statistics.

Out of 84 diabetic patients, 17 (20.2%) have Hp1-1 phenotype, 34.6% have Hp2-1 phenotype and 45.2% have Hp2-2 phenotype. Among the control group, 51 (60.7%) have Hp1-1 phenotype, 16.7% have Hp2-1 and 22.6% have Hp2-2 phenotype.

Odd ratio test was carried out to check the tendency of the diabetic Hp phenotypes to develop ACR. An odd ratio value of 0.150 with no statistical significance (p= 0.152) was obtained for Hp 1-1 phenotype, odd ratio value of 0.273 and a p value of 0.064 was obtained for Hp 2-1 phenotype and odd ratio value of 0.016 and p value of 0.003 was obtained for Hp2-2 phenotype. This shows that there is no tendency of diabetic patients with Hp1-1 and Hp2-1 phenotype to develop ACR. However, there is tendency of diabetic patients with Hp2-2 to develop ACR.

Table 1 below showed the distribution of haptoglobin phenotypes among the study population.

Table 1: Frequency Distribution of Haptoglobin Phenotypes among the Study Population

Haptoglobin Phenotype	Test Group (%) (n=84)	Control Group (%) (n=84)
Hp1-1	17(20.2)	51(60.7)
Hp2-1	29(34.6)	14(16.7)
Hp2-2	38(45.2)	19(22.6)
Total	84(100)	84(100)

Table 2 below explained the comparison of mean values of parameters between the Hp phenotypes.

Table 2: Comparison of Mean Values of Parameters between the Hp Phenotypes

Parameter	Hp1-1	Hp2-1	Hp2-2	P value
Creatinine (µmol/L)	62.65±10.03	75.97±59.28	60.50±10.11	0.199
BMI (Kg/m^2)	26.21±6.34	23.60±4.70	25.51±4.90	0.184
FBG (mmol/L)	11.84±6.48	11.89±6.06	11.34±5.89	0.923
HbA1c (%)	8.58±2.04	8.70±2.68	8.24±1.96	0.687
DD(Years)	7.58±5.62	5.59±5.31	6.15±6.34	0.538
Urea(mmol/L)	3.84±1.52	4.32±2.56	3.60±1.30	0.303
ACR(mg/g)	19.29±9.16	37.24±29.03	23.11±21.94	0.007

BMI=body mass index FBG=fasting blood glucose HbA1c= glycatedhaemoglobin DD=duration of disease ACR= albumin creatinine ratio

Table 3 showed the odd ratio of developing microalbuminuria among the haptoglobin phenotypes.

Table 3: Odd Ratio of Developing Microalbuminuria among the Haptoglobin Phenotypes

	No. Tested	ACR(mg/g)		OR	95% CI	P-Value
		<30	30 - 300			
Hp 1 – 1						
Diabetic	17	15 (88.2)	2 (11.8)	0.150	0.013 – 1.771	0.152
Control	51	50 (98.0)	1 (2.0)	0.232	0.053- 1.987	0.224
Hp 2-1						
	29	18(62.1)	11(37.9)	0.273	0.033-2.659	0.064
	14	12(85.7)	2(14.3)	0.371	0.065-2.796	0.503
Hp2-2						
	38	32(84.2)	6(15.8)	0.016	0.033-2.659	0.003
	19	18(94.7)	1(5.3)	0.371	0.065-2.796	0.503

Figure 1 showed the Gel electrophoresis of amplified DNA products showing PCR results of the haptoglobin genotyping of patients with type 2 diabetic patients.

Discussion

Diabetic nephropathy is one of the complications of diabetes mellitus of public importance. Early detection of diabetic nephropathy is of paramount importance in order to provide appropriate therapy that prevents or slows evolution towards end stage renal disease. In early detection of diabetic nephropathy, biomarkers play an important role and the best known is microalbuminuria testing. Microalbuminuria (MA) is an early marker of nephropathy[2]. Therefore, early detection and appropriate interventions in asymptomatic individuals may help in preventing deterioration in renal function progression to diabetic nephropathy and end stage renal disease[3].

Genetic differences between diabetic patients might play an important role as to the reason why some develop complications and others do not[10]. Diabetes is accompanied by state of severe oxidative stress. The stress is released by lipid peroxidation arising from an excess of free reactive oxygen species intermediate (ROS). Oxidative stress and antioxidant imbalance caused by diabetes mellitus is a predispositing factor for the development of diabetes complications with nephropathy inclusive. Haptoglobin (Hp) molecule is the counterpoint in the body to these ROS. It is well established that the functional properties of haptoglobin are type dependent.

The main function of haptoglobin is binding to haemoglobin (Hb) so as to prevent haemoglobin induced oxidative tissue damage. The antioxidant effect of haptoglobin is mediated in part by the ability of haptoglobin to prevent the release of iron from haemoglobin on its binding[11]. The haptoglobin gene has three phenotypes mainly Hp1-1, Hp2-1 and Hp2-2. Hp binds and aids in the clearance of free Hb

stemming from low grade intravascular haemolysis[5]. Kidney damage can be prevented by passing free Hb in circulation through glomerular filterate. The present study demonstrated the frequency of Hp 1-1, Hp2-1 and Hp2-2 in type 2 diabetic patients to be 20.2 %, 34.6 % and 45.6 % respectively. Olayinka and Humphrey[12] supported the finding of the current study. Based on the current finding and in respect to MA, Hp1-1 was demonstrated to be a preventive factor owing to the fact that most of the Hp1-1 phenotype diabetic patients had a normal ACR. This is actually supported by the findings of Awadallah and Hammad[13], Burbea *et al*.[14] and Bessa *et al*.[15]).

Hp1-1 binds more strongly to free Hb and also a better antioxidant. The antioxidant function of Hp1-1 is thought to confer protection from angiopathies. Hp1-1 is considered to be small and penetrate well due to its size. The relative protection afforded by the different Hp phenotypes can be the result of difference in molecular shape and size between the protein products encoded by two different Hp allele. Smaller Hp complexes penetrate into the extracellular spaces to undergo glomerular sieving. The current study revealed no statistical significance between the ACR of diabetic Hp1-1 and control Hp1-1. This implies that most of diabetic Hp1-1 had a normal ACR. It has been observed in different studies of haptoglobin antioxidant property that susceptibility to the development of vascular complications has different degrees; with Hp1-1 individuals showing better protection against diabetic nephropathy than other phenotypes. This variation is not attributed to difference in affinity between the haptoglobin subtypes and Hb molecule but rather reflected to the fact that Hp1-1 pass more easily through the endothelial barrier to reach the extravascular spaces because of its small size and structure.

Therefore Hp1-1 individuals are better protected against oxidative stress[16,11,5]. This is also supported by Quaye *et al*.[17] in Ghanian population, Bessa *et al*.[15] in Egyptian population, Awadallah and Hammad[13] in Mongolian Brazilian and Jordanian study and Shi *et al*.[18] in Northern Han Chinese.

Also, the current study shows that there is no statistical significance when the ACR of the both the diabetic and control group of the Hp1-1 phenotype were compared. This implies that both the control and diabetic patients with Hp1-1 phenotype have a normal ACR level. Therefore, the fact that Hp1-1 phenotype provides a better protection is proven by the current finding. Many studies on haptoglobin antioxidant property have shown that susceptibility to diabetic vascular complications have different degrees with Hp1-1 showing better protection than Hp2-1 and Hp2-2[16,11,5]. Hp1-1 is a better antioxidant with more affinity to free Hb than Hp2-2[19].

Findings from the studies of Nakhoul *et al*.[16], Asleh *et al*.[11] and Levy *et al*.[5] are consistent with the current finding as shown by the odd ratio that Hp1-1 is not in any way a risk factor for the development of MA in type 2 diabetic patients.

Goldenberg-Cohen.[20] carried out a research and found that Hp1-1 phenotype did not appear to have an implication on the risk of diabetic nephropathy. The findings of Asleh,[11] and Adinortey *et al*.[21] are also consistent with the current finding. All the studies have attributed the phenomenon to the protective function of Hp1-1. Kidney damage can be prevented by passing free Hb in circulation through glomerular

filterate. Many studies on haptoglobin antioxidant property have shown that susceptibility to diabetic vascular complications have different degrees with Hp1-1 showing better protection than Hp2-1 and Hp2-2[16,11,5]. Hp1-1 is a better antioxidant with more affinity to free Hb than Hp2-2[19].

Hp1-1 has been demonstrated to provide superior protection against Hb-iron driven peroxidation than Hp2-2. The superior antioxidant capacity of Hp1-1 seems not to be related with a dissimilar affinity with Hb but the functional differences may be explained by the restricted distribution of Hp2-2 in extravascular fluids as a consequence of its high molecular mass[19]. The protein product of the Hp 2 allele has an inferior ability to bind to hemoglobin. This may lead to iron overload during periods of hemolysis and has been linked with renal injury. In addition, the Hp 2 allele product is also an inferior antioxidant compared with the Hp 1 allele product, which can further exacerbate tissue injury[22]. The relative protection afforded by the different haptoglobin phenotypes can be the result of differences in the molecular shape and size between the protein products encoded by two different haptoglobin alleles[16].

Several studies have linked the Hp2-1 and Hp 2-2 Phenotypes with various complications of diabetes mellitus including nephropathy. In this study, patients with Hp 2-1 genotype were not found to have an increased risk of developing diabetic nephropathy where as those with Hp2-2 were found to have an increased risk of developing diabetic nephropathy. This agrees with the findings of Vasudevan *et al.*[23]in India, who found an increased risk of developing diabetic nephropathy in patients with the Hp 2-2 genotype. Olaniyan *et al*.[24] found higher incidence of nephropathy among patients with Hp 2-2 genotype than other Hp genotypes in Nigeria. This is similar to the findings of Roy *et al.*[25]in India, who found a 1.89 times risk of developing nephropathy in patients with Hp 2-2. Adoinortey *et al*.[21] carried out similar research on Ghanian population and is also in consistent with the current finding. Hp2-2 may therefore be useful as a predictor for diabetic individuals to develop diabetic nephropathy. It would therefore be worthwhile for individuals to know their Hp phenotype so as to adapt lifestyle that would delay the onset of diabetic nephropathy. However, Awadallah *et al.*[26] in Jordan reported that Hp2-2 is not a risk factor to the development if diabetic nephropathy and therefore in disagreement with the finding of this study. The current study observed no statistical significant difference in the comparison of the biochemical parameters of all the Hp phenotypes of diabetic groups except for ACR. The current finding agrees with the findings of Awadallah *et al.*[26] in Jordan. However, Olaniyan *et al.*[24] and Nakhoul *et al.*[16] in Israel reported difference between fasting blood sugar and HbA1C of Hp 2-2 diabetics and Hp 1-1 diabetics in Nigeria. The current study revealed statistical significance between the ACR of diabetic Hp2-1 and Hp2-2 were compared with their corresponding controls Hp. This implies that most of diabetic Hp2-1 and Hp2-2 had more of microalbuminuria.

Conclusion

This study demonstrates that the haptoglobin genotypes Hp1–1, Hp2–1, and Hp2–2 are present among the studied population of patients with type 2 diabetes mellitus. While the Hp1–1 and Hp2–1

polymorphisms were not associated with an increased risk of microalbuminuria, the Hp2–2 polymorphism showed a significant association with the development of microalbuminuria in patients with type 2 diabetes. These findings suggest that the Hp2–2 genotype may serve as a genetic risk factor for early diabetic nephropathy, highlighting its potential relevance in risk stratification and targeted preventive strategies in diabetic care.

Declarations

Ethics approval and consent to participate

This research was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki for research involving human participants.

Ethical approval for the study was obtained from the Health Research Ethics Committee of the participating institution(s) in Kano State, Nigeria. Permission to conduct the study was also obtained from the management of the healthcare facilities where participant recruitment and sample collection were carried out.

All eligible participants were adequately informed about the objectives, procedures, potential risks, and benefits of the study in a language they clearly understood. Participation was entirely voluntary, and written informed consent was obtained from each participant prior to enrolment. Participants were assured of the confidentiality and anonymity of their personal and clinical information, and data were coded to prevent identification of individual participants.

Participants were informed of their right to decline participation or withdraw from the study at any stage without any consequences to their medical care. Biological samples collected were used strictly for the purpose of this study, and no additional analyses were conducted without prior consent.

Consent for Publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included within the main article.

Competing Interests

The authors declare that they have no competing interests.

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Figures

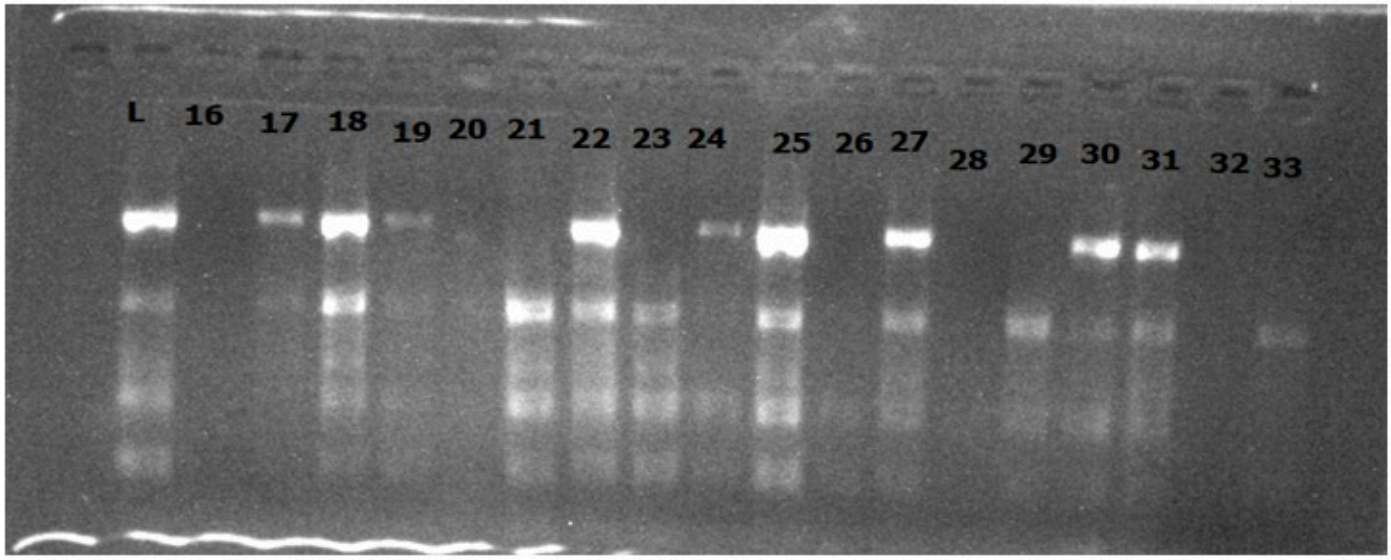


Figure 1

Gel electrophoresis of amplified DNA products showing PCR results of the haptoglobin genotyping of patients with type 2 diabetic patients. Lane L is 200bp DNA ladder, Lane 16 and 20 are negative controls, Lane 18-32 are samples and Lane 17 and 33 is positive controls. Lane 23 and 29 are Hp1-1 while 19, 24, 27, 30 and 31 are Hp2-1

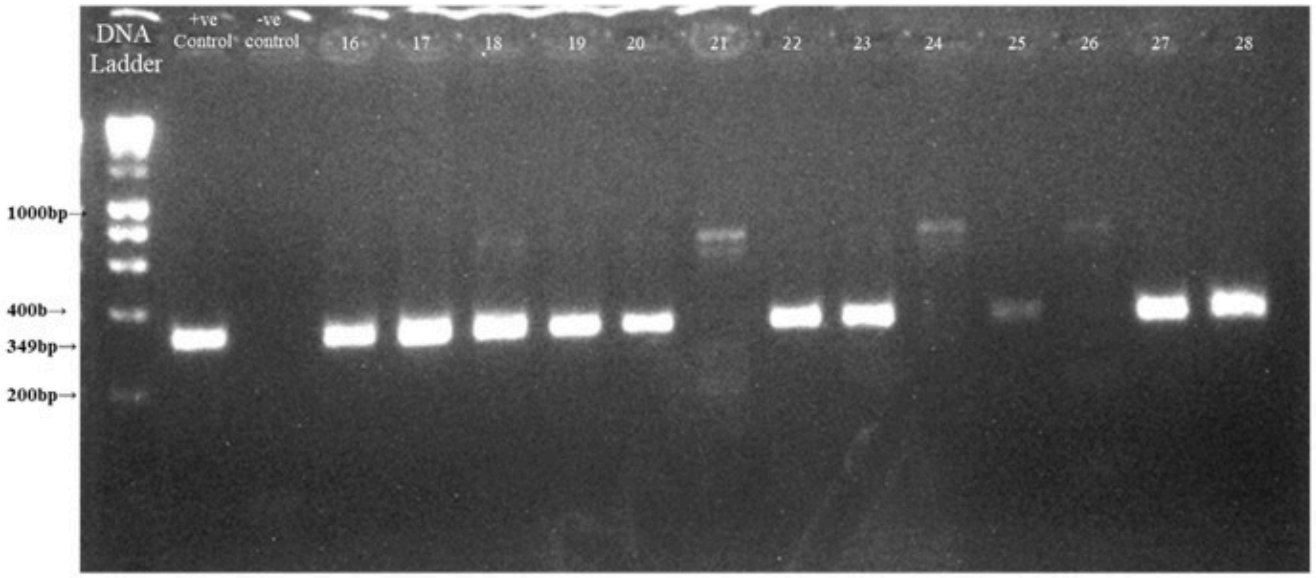


Fig.1: Lane1-200bpDNA Ladder, Lane 2-Positive Control, Lane 3- Negative Control, Lane 16-28 Samples

Figure 2

See image above for figure legend.