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RESEARCH

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Association of ADAM33 gene with COPD pathophysiology: a case–control study

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Abstract

Background Worldwide, Chronic Obstructive pulmonary disease (COPD) is a main cause of morbidity and mortality. Considering the global increase in the prevalence of COPD, research on the genetic factors that predispose to COPD is reviving. Recently, ADAM 33 has been found to be related to severe lung function decline and COPD.

Aim and objective The present study is carried out with the main aim of determining the association of SNP, i.e., S2 (rs528557), with COPD.

Method A case–control methodology is used to recruit participants. 50 COPD patients over 40 years of age and with a history of more than 20 pack years of cigarette smoking were enlisted. The same number of age and gender-matched controls with no COPD history were involved. PCR sequencing was used to analyze the genetic polymorphism of the ADAM 33 gene (SNP, i.e., S2 (rs528557). Statistical analysis was carried out using SPSS version 21. The Chi-square test was used to determine the difference in SNP rs528557 genotypes and alleles between controls and COPD.

Results The findings of this study revealed that the G allele was present in all COPD cases (100%) and 72% of control ($p = < 0.001$). The minor C allele was 14% and 32% in COPD patients and control, respectively. The G/G genotype is overrepresented in cases (25.5%) than in the control (9.2%). The C/C genotype is overrepresented in controls (3.8%) than in COPD patients (0.9%).

Conclusion The findings of this study demonstrate a significant association of the ADAM 33 gene (SNP, i.e., S2 (rs528557) with COPD pathophysiology in the studied group.

Keywords COPD, ADAM33, Genetic polymorphism, Cigarette smoking, SNP

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Introduction

Although worldwide, COPD is the third major cause of mortality [1, 2]. In 2019, almost 456 million COPD cases and 4 million deaths were reported globally [1]. This disease is mainly characterized by limited airflow and the lung's abnormal inflammatory response to harmful gases and particles, particularly cigarette smoke [2]. These substances usually lead to airway remodeling and emphysema, manifested by epithelium mucous and squamous metaplasia, fibrosis of the airway wall, and hypertrophy of smooth muscles. This leads to the progressive obstruction of airflow [2, 3]. COPD is a significant public health issue that has a damaging financial impact. It is estimated that by 2030, the COPD worldwide cost will reach $4.9 trillion [4]. In addition, the comorbidities such as mental health, lung cancer, and cardiovascular disease related to COPD would increase the burden on the health system [3].

COPD is multifactorial comprising complex inflammatory cells, such as lymphocytes, neutrophils, monocytes, and eosinophils. In addition to the inflammatory cell, increased cytokine levels such as VEGF, TNF-a, IL-8, and IL-6 also play a significant role in COPD pathogenesis [5]. The role of environmental factors in COPD development is well documented [6]. Cigarette smoking is the major risk factor for the development of COPD. Shisha and passive smoking are other COPD risk factors [6, 7]. COPD is also attributed to occupational exposure to outdoor pollutants and inorganic and organic dust. The prevalence of COPD in smokers is 12–15% which shows that some underlying genetic factors have a main role in COPD development and severity [7, 8]. Unlike asthma, the inflammatory mediators that have a role in the pathogenesis of COPD are lipid mediators, ROS, cytokines, inflammatory peptides, growth factors, and chemokines, which cause alveolar destruction and airway fibrosis. The protease role in the extracellular matrix and imbalance in the pulmonary emphysema genesis is well accepted [9]. Other risk factors include lower socioeconomic status and genetic factors. Genetic factors mainly predispose some people to tobacco exposure for respiratory disease development. ADAM proteins have a role in cell fusion, adhesion, proteolysis, and signaling. ADAM 33 (a disinterring and metalloprotease) is an active protease that has the ability to split synthetic peptides and α2-macroglobulin [9]. The ADAM 33 is mainly involved in tissue remodeling and pulmonary defense. Chronic pulmonary diseases such as asthma and COPD are mainly characterized by persistent inflammatory processes and airflow obstruction. The process of inflammation occurs due to the complex interaction between inflammatory mediators and cellular mechanism and their impact [9]. ADAM 33 is an active protease that is involved in the α2-macroglobulin cleavage, a pulmonary defense system member [10]. This suggests that ADAM33 is involved in airway obstruction pathogenesis, impacting tissue remodeling and leading to airway inflammation. It has been demonstrated that within ADAM33, single nucleotide polymorphisms (SNPs) are related to increased lung function decline [10]. The ADAM33 gene comprises 179 single nucleotide polymorphisms loci (SNP). The polymorphisms of the S2 locus present in the ADAM33 have been related to the COPD development [10]. To our knowledge, previously, no study has been carried out in Pakistan that has determined the association of SNP i.e., S2 (rs528557), with the COPD risk. Therefore, this study is conducted with the main aim to determine the association of SNP, i.e., S2 (rs528557) with COPD.

Methodology

Study design and population

This is a case–control study that was endorsed by the Ethical Committee of Islamia University Bahawalpur Informed consent was taken from all participants who were willing to participate in this genetic study. In this study, 50 COPD patients over 40 years of age and with a history of more than 20 pack years of cigarette smoking were involved. The same number of age and gender-matched controls with no COPD history were involved.

Inclusion and exclusion criteria

The inclusion criteria for cases include ≥ 20 pack years of smoking history; age 40 years or above, and COPD (dyspnea, hack, sputum) and post-bronchodilator constrained expiratory proportion (FER) or FEV1/FVC under 0.7 (70%). Controls were matched for gender, age and, smoking years with cases and asymptomatic for chronic respiratory disease. The exclusion criteria for cases and control include known diseases of psoriasis and asthma and a family history of both these diseases.

Data collection

The questionnaire was used to collect detailed information about person and family history, employment status, and smoking history. The questionnaire was reviewed by the physician at the examination time. All participants were asked to mention their cigarette smoking as packs/day and patient proforma was filled. Weight and height were measured and blood samples were taken. Spirometry was done for the COPD confirmation according to the international criteria. Residual volume (RV) was found in a subset of the study population (FVC ≤ 80% predicted) to confirm hyperinflation or restriction presence. History of dyspnea, breathlessness or sputum creation, as well as history of exposure to risk factors for the illness was collected. Spirometry in the presence of a FEV1/FVC < 0.70

affirms the COPD. The disease seriousness was graded by GOLD guidelines.

DNA extraction protocol

The DNA was extracted from samples by using the Thermo Scientific Gene Jet Genomic DNA purification kit LOT#00834919. The S2 region was amplified for cloning with the help of polymerase chain reaction (PCR)kit THERMOSCIENTIFIC PCR master mix (2×) LOT#=00785324. The primer sequence is shown in Table 1. The final amplified product comprises 304 base pairs and was run through Gel electrophoresis to isolate the DNA fragments based on molecular weight to get the band and visualized under UV light. The DNA of 100 samples was isolated and analyzed in 0.8% agarose gel.

PCR results

Successfully amplification was carried out for each of the three primers pairs. PCR products were visualized in 1% agarose gels. For primer pair S2 (rs528557), bands of 350 base pairs were amplified successfully. These base pairs were restricted as follows: For the GG genotype, three bands of 54, 42 and 19, 19bp and 96bp for the C Genotype, and all four bands for the heterozygous genotype G/C. Purified PCR products of the S2 primer pair (350bp) were digested by the enzyme Fsel.50 base restriction products present in all three genotypes bands have 'run out of the gel during the electrophoresis process. This enzyme recognizes the restriction site GGCCGG/CC. (Please see the Appendix for the detailed procedure) (Figs. 1, 2, 3, 4, 5).

Table 1 Show DNA with polymerase chain

ADAM33	S2	REVERSE:5′-AGAGCTCTGAGGAGGGGAAC-3′ FORWARD: 5′-TGTGCAGGCTGAAAGTATGC-3′

Statistical analysis

The data were analyzed by using SPSS version 21. The significant value was 0.05. Mean and standard deviation were found for numerical data, whereas frequencies and percentages were calculated for qualitative variables. The Chi-square test was used to determine the difference in SNP rs528557genotypes and alleles between controls and COPD. An online calculator was used to detect any significant deviations from Hardy Weinberg equilibrium based on Chi-square test. *T* test was used to compare pack years, age and BMI between cases and controls considered.

Results

The sample of participants is divided into four subgroups based on GOLD guidelines to determine the effect of genetic variation on the disease progression. In

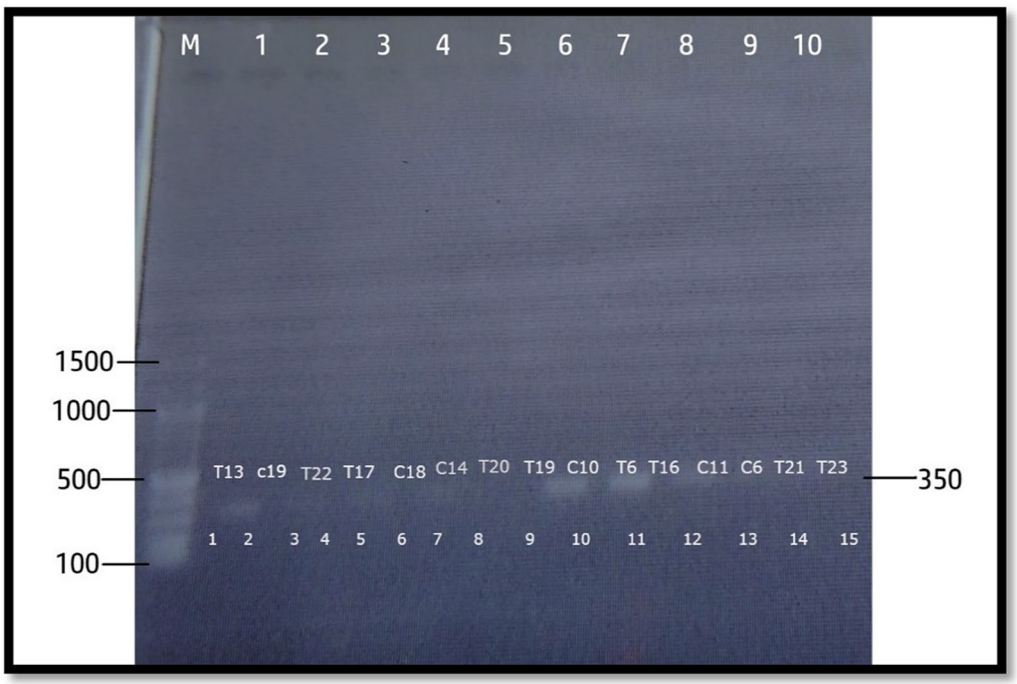

Fig. 1 1% agarose gel electrophoresis of PCR product showing; Lane 1 contains DNA ladder. Lane 2 contains DNA band; Lane 3 contains no band and Lane 4, 5, 6 contain band; Lane 7, 8, 9 contains no bands but 10 contain 10 Kb DNA band

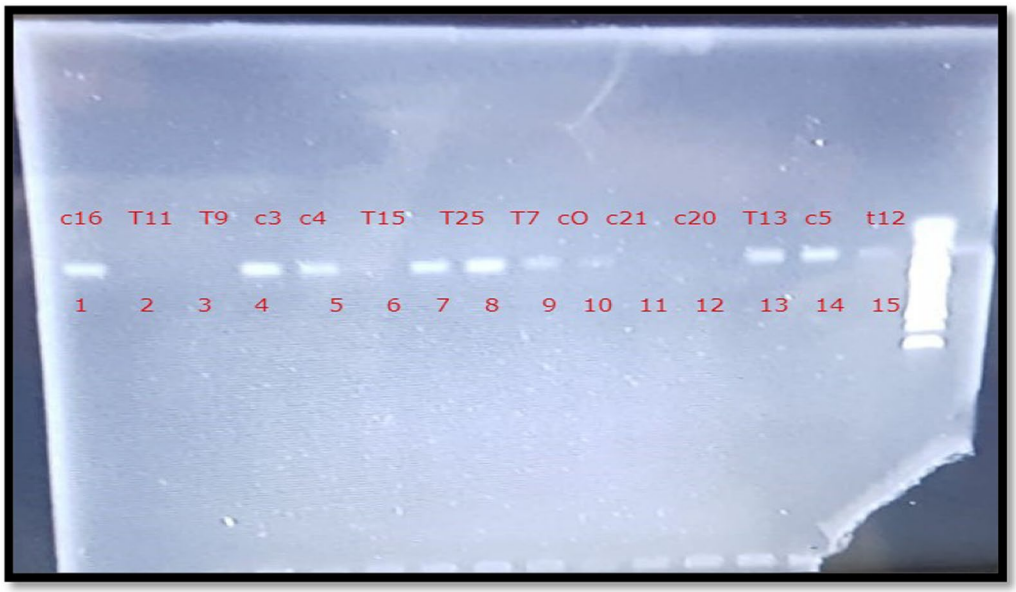

Fig. 2 From left to right: Lane 1 contain DNA ladder, Lane 2, 3, 4, 6, 7, 8, 9, 11 and 12 contains bands and Lane 5, 13, 14, 15, 16 contain no band showing 1%gel electrophoresis PCR product

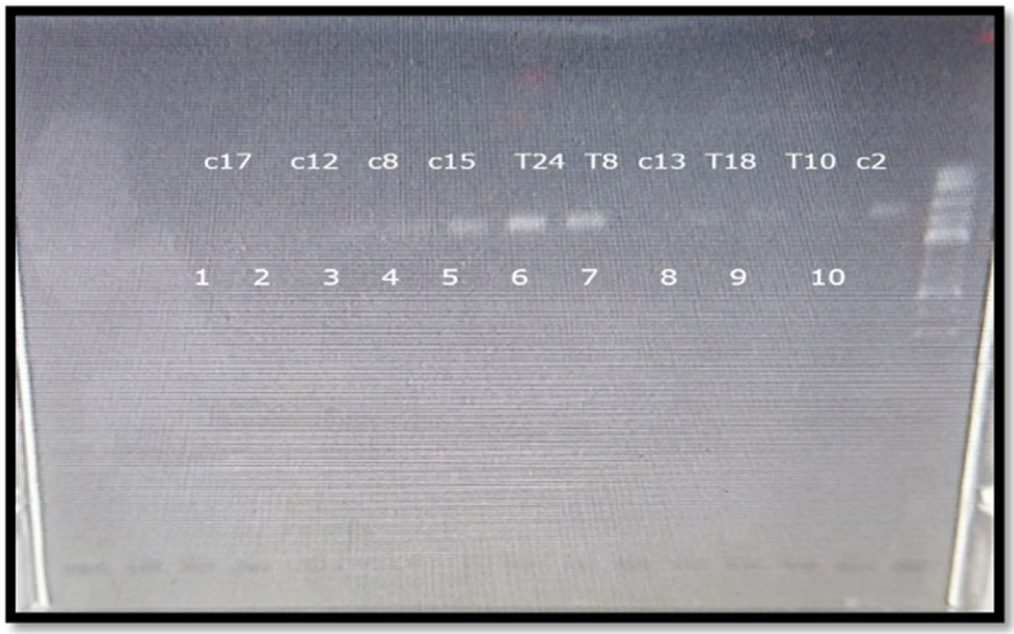

Fig. 3 1% Agarose gels electrophoresis of PCR product shows bands from left to right. Lane 2, 3, 4, 5, 7, 8, 9, 10 except 6 not showing band. (Due to poor picture quality, the bands were noted manually)

this study, a total of 50 asymptomatic gender and age-matched controls were recruited. The clinical and demographic data of healthy controls and samples under this study are shown in Table 2. The mean age of controls was 51.08 + 8.03 years, and the mean age of COPD patients was 56.71 + 11.87 years. Among COPD patients, all were found to be smokers, whereas among control 10 were smokers and 40 were nonsmokers. Among controls, the average smoking history was 4.8 pack years, whereas in COPD patients it was 23.5 pack years. A significant difference has been found between breathlessness, age, pack years, and BMI between cases and control (Table 2).

Fig. 4 Agarose gel electrophoresis results showing results of RFLP with showing Lane 1 DNA ladder, Lane genotype 4, 6, 10, 13, 14 and 16 represent Homozygote GG, Lane 8, 11, 12 represents Heterozygotes CG and Lane 5 and 9 represents Homozygotes CC

Fig. 5 2% Agarose gel electrophoresis results showing results of RFLP. Lane upper and lower 1, 4, 5, 6, 7, 12, 16, 19, 20, 21, 22, 25 represent Homozygote GG genotype and lane 2, 8, 10, 15, 17, 18 represents Heterozygote CG and Lane14 and 18 represents Homozygote CC

Genotype analysis

The findings of genotyping have revealed the frequencies of alleles and genotypes as shown in Table 3. The G/G genotype is overrepresented in cases (25.5%) than the control (9.2%). Among cases, the genotype distribution is in line with Hardy Weinberg equilibrium ($p=0.56$) but significant deviation has been seen in control ($p=0.09$). G allele was found to be overrepresented in COPD patients, whereas allele C is dominant in the control group. Genotypic frequencies of ADAM33 gene SNP rs528557 were found to be GG/CC/CG 70.6%, 3.9%, 25.5% in COPD patients while they were found to be 88.2%, 9.2%, 0.8% in healthy controls, respectively. The G/G genotype of SNP rs528557 is found to be overrepresented in COPD patients (70.6%) than control (86.9). The C/C genotype is found

Table 2 Demographic and clinical data of the participants

Characteristics	COPD $n=50$	Controls $n=50$	p value
Age years (mean ± SD)	56.71 + 11.87	51.08 + 8.03	0.01*
Gender *n* (%)	Males = 41 Females = 9	Males = 24 Females = 26	< 0.001**
BMI KG/M2 (mean ± SD)	32.26 ± 9.07	37.35 ± 7.15	0.02*
Smoker/non-smoker	50/0 100/0.0%	10/40 20/80%	< 0.001**
Pack years	23.5 ± 1.58	4.80 ± 9.78	< 0.001**
Presence of chronic cough	50 (100%)	46(92%)	0.04*
Diabetes mellitus	11	15	0.19
Breathlessness	48	39	0.01*
Osteoporosis	0	8	0.01*
Cardiac disease	6	3	0.40
Sputum production	45	28	< 0.001**
Psychological disorders	3	4	0.64
Hypertension	12	4	0.02*
Frequency of C allele	7 (14.0) %	16 (32.0) %	0.03*
Frequency of G allele	50 (100) %	36 (72.0) %	< 0.001**

* less than 0.05, ** less than 0.001

Table 3 Represents statistical calculations and expressing numbers

Genotypes	GG	GC	CC	p value
Cases	36 (70.6)	13 (25.5)	2 (3.9)	0.56
Control	45 (88.2)	5 (9.2)	1 (0.8)	0.09

to be overrepresented in controls (3.8%) than COPD patients (0.9%).

Discussion

COPD is mainly characterized by airflow restriction due to emphysema or obstructive bronchitis, thus leading to pulmonary failure. To determine the genetic variance association in COPD, ADAM33 gene SNP rs528557 was genotyped and analyzed in the Pakistani population by using the case–control methodology.

ADAM 33 is characterized as Zn + 2-dependent metalloproteinases with different functions and encompass signal transduction. A total of seven domains are present in ADAM33, including a pro-domain, a catalytic domain, a metalloprotease domain, a disintegrant domain (which interacts with integrins), a transmembrane domain, and a cytoplasmic domain [11, 12]. Previous studies have associated the polymorphism in ADAM33 in COPD patients [13–16]. Studies have found ADAM 33 expressed in smooth muscle cells of the airway and lung fibroblasts. ADAM 33 also plays the main role in airway remodeling due to its high expression in fibroblast and smooth muscle cells. As higher ADAM 33 expression has been found in lung smooth muscle and fibroblast, the ADAM 33 under and over expression usually results in changes in airway remodeling and repair process [13–16]. Considering this, the present study is carried out to concentrate on the ADAM 33 gene polymorphism and its role in COPD. The findings have shown that polymorphism impacts COPD, which may be due to ethnic variation and genetic background. It has been demonstrated that SNPs located in the ADAM 33 gene functional domain mainly contribute to the expression and transcription of the ADAM 33 proteins and mRNA. The expression of these proteins then influences the ADAM 33 function in COPD pathogenesis. S2 encodes Gly717Gly, which does not alter the amino acid sequence but changes the translation process, m RNA stability and mRNA folding. There is a need for further studies to determine the S2 variation impact on the function of ADAM33.

The findings of this study demonstrated a significant association between age and COPD patients. This shows that older people are more vulnerable to COPD. The aging process is mainly characterized by the increased rigidity of the chest wall, reduced lung recoil elasticity and loss of power of respiratory muscles responsible for the loss of function of FEV1 and increased residual volume. This finding is also in line with the previous studies in which it has been demonstrated that changes in lungs structurally and functionally over age made the lungs more vulnerable to the development of COPD [9]. In the current study, males were overrepresented, and a strong association has been found between gender and susceptibility to disease, which is in accordance with the findings [17]. Through the history evaluation regarding symptoms, it was found that 96.1% of individuals presented chronic cough symptoms and 88.2% with breathlessness; however, a smoking history was found in 60.8% of patients, which is in contrast to other western studies where no significant difference have been found between gender and COPD [15]. In the current population, the prevalence of smoking is higher in males, which could support the increased ratio of COPD in men as compared to females. The present study also showed that 22 Packs per year of smoking ($p=<0.001$) is significantly associated with COPD, which explains the fact that the intensity of smoking is also associated with the development and severity of the disease, which is also evident from previous studies [12, 15]. Tobacco smoking is highly associated with chronic sputum production, which aggravates bronchial infection leading to alveolar and airway damage that could result in airflow restriction. Breathlessness and Chronic sputum

production are also significantly associated with smoking. Excessive production of sputum declined the FEV1 and raised the hospitalization risk due to COPD which supports the finding of the current study clearly showing the significant association of breathlessness and chronic sputum production in patients suffering from COPD as compared to a healthy individual. The present study described the COPD association with lower body weight, undernutrition due to chronic inflammatory processes, dyspnea, diabetes mellitus, hypertension, cardiac illness and osteoporosis and found that except DM and cardiac disease, hypertension, lower body weight, dyspnea and osteoporosis are significantly associated risk factors of COPD. These findings are in line with the previous studies [12, 15, 18].

The present study has demonstrated a lower frequency of the minor SNP S2 in the Pakistani population compared to the major allele in patients of COPD when compared with healthy controls that are comparable with previous studies [12, 15]. Figarska et al. (2013) study analysis showed an association of the ADAM33 gene with COPD pathophysiology and showed that carriers of minor alleles of SNPs Q-1, S1, S2, and T2 have higher mortality due to COPD [19]. Overproduction of ADAM33 leads to the release of inflammatory mediators and growth factors inducing pathological changes. In the current study, S2 SNP is shown to be associated with COPD. S1 and S2 SNPs are located in exon S, but is involved in the modification of the structure of the protein. The ADAM33 SNP, in particular, is related to a greater decline in FEV1 in general population samples. In the current study, the S2 (rs528557) SNP is associated with COPD and has a higher frequency of major allele frequency in patients suffering from COPD than controls. It was observed that ADAM33 multidomain gene polymorphism is a susceptibility gene for COPD, which is line with the previous studies [12, 15, 18, 20, 21]. Yuan et al. (2017) demonstrated that 88 smokers were genotyped for 25 ADAM33 single nucleotide polymorphisms [22]. The current study, as compared to the above, shows a more significant relation of ADAM33 gene polymorphisms of SNP S2 with COPD.

The study has several limitations. Only S2 was included. The inclusion of other S1 could have improved the finding of this study. The results of the current study are diverse compared to previous research might be due to different ethnicities. Moreover, the sample size is limited in our study. More comprehensive data should be obtained for in-depth mechanisms and making clear interpretations regarding the current association. Other polymorphisms of ADAM33 can also be included in this potential panel of SNPs so that patients at risk of COPD may be identified in the future. However, despite this, the findings of this study provide useful insight into the association of SNP i.e., S2 (rs528557) with COPD.

Conclusions

The findings of the present study are the first to reveal a significant association of SNP i.e., S2 (rs528557) with the COPD pathophysiology in the studied population. The contribution of SNP in COPD pathophysiology needs to be clarified yet. Future studies should be carried out to expand this preliminary research with a large sample size and other SNPs to fully understand the potential ADAM 33 genes in the COPD genesis in the Pakistani population.

Appendix

DNA extraction protocol procedure

Using the ThermoScientific GeneJet Genomic DNA purification kit LOT#00834919, DNA was extracted from samples by following its protocol as follows: Samples were labeled as cases (CS1-CS51) and controls (CT1-CT51) and added with lysis solution 400 µl and 20 µl of Proteinase K solution into 200 µl of blood through the use of a pipette. Then, were vortexing to make a uniform solution. Samples were incubated at 56 °C for 10 min to lyse cells completely. Then, 200 µl ethanol (96–100%) was added and vertex. The prepared lysate was transferred to the purification column of Genome Genejet Genomic DNA and then collected in the collection tube.

Centrifugation was done for 1 min at 6000×*g*, and the flow through solution was discarded. In 2 ml collection tube, the column was placed. 500 µl wash buffer 1(added ethanol) was added, and the centrifuge was done for one minute at 8000×*g*. After this again, flow through solution was discarded and in the collection tube, purification column was placed. 500 µl of Wash Buffer II (with ethanol added) was added to the Purification Column of GeneJET Genomic DNA. Centrifugation was done for three minutes at 12,000×*g*, the flow through solution was discarded, and Purification Column was placed in a Sterile 1.5 µl centrifuge tube. 200 µl of elution buffer was added to the purification column. Incubation was done for two minutes at room temperature and centrifugation for one minute at 8000×*g*. The purified DNA was stored in Eppendorf's at −20 °C after discarding the purification column.

Gel electrophoresis for DNA

GEL electrophoresis requirements

0.8% gel and a 1 kb DNA ladder were used for DNA isolation. The gel was prepared as follows:

- Agarose Gel
- Electrophoresis buffer
- DNA Ladder
- DNA samples
- Power supply
- Gel-using trays (made of UV transplant plastic)
- Comb (to form wells for loading sample)
- Electrophoresis chamber
- Loading dye (contain glycerol to allow the sample to fall in the wells properly and tracking dye allows for visualization or to monitor the electrophoresis proceedings

Agarose gel preparation

The comb was fixed, and steel sliders were in casting trays; poured the gel in a tray up to 3–5 mm thick. Then, allowed to solidify for 20 min, mounted the gel on the electrophoresis chamber with 1× buffer, added enough to dip the gel to 1 mm, and carefully removed the comb. A mixed 5 µl DNA sample with 2 µl loading dye (6×) was loaded in the wells using a micropipette. Loaded 1.5 µl DNA ladder in the last wells and closed the lid. The electric leads were attached to the negative and positive terminals of the tanks and passed 3 V/cm voltage. The bromophenol blue was allowed to run up to the appropriate distance through the gel and stop the flow of current (Voltas, 2000).

Staining and visualization

The gel from electrophoresis was stained in a solution containing ethidium bromide. The working solution contained 0.01 g Ethidium powder in 1 ml distilled water was prepared. 75 µl of it was mixed in 500 ml of distilled water and used for staining. The gel was submerged in the solution for 25–30 min. Carefully recovered the gel from the stain, washed it under tap water, and visualized it in the gel documentation system under UV illumination. The image was recorded and saved.

Polymerase chain reaction (PCR)

The S2 region was amplified for cloning with the help of polymerase chain reaction (PCR)kit THERMOSCIENTIFIC PCR master mix (2×) LOT#=00785324. The primers were designed to partially amplify the S2 sequence from base to base of genome segment A. The start codon in the forward primer is underlined. Samples were amplified using cDNA as a template, and a PCR reaction was set up, as shown in the table below. During all the procedures, cDNA and PCR reagents were kept on ice. All the reagents for PCR were mixed in a 0.5 ml centrifuge tube,

Table 4 Shows reagents and their respective qualities

Sr. no.	Reagent	Quantity
1	PCR Master Mix (2×)	25 µl
2	Forward primer	1.5 µl
3	Reverse primer	1.5 µl
4	Template DNA	5 µl
5	Water, nuclease-free	17 µl
Total		50 µl

Table 5 A summary of PCR methods and their associated conditions

Step	Temperature °C	Time	Number of cycles
Initial Denaturation	94	5 min	1
Denaturation	94	30 s	40
Annealing	60.45	30 s	
Extension	72	1 min/kb	
Final Extension	72	10 min	1

short spun and set in a thermal cycler (ESCO-ARIES) under the following conditions to amplify the nucleic acid.

Protocol

After labeling the PCR tubes according to samples, in each sample, vials added 25 µl of thermos scientific kit master mix (2×) after gentle vertexing and thawing. Each component was added according to the above table, maintaining temperature in the cabinet for each 50 µl reaction. 1.5 µl forward and reverse primers were added to each PCR tube. 5 µl of the DNA template and 17 µl of nucleus-free water were added to each PCR tube to make the volume up to 50 µl. The sample was gently vortex and spun down. The thermal cycler overlay the reaction mixture with 25 µl of mineral oil that does not contain a heated lid. PCR was performed under following conditions.

After completion of the process, the final amplified product was run through Gel electrophoresis to separate the DNA fragments based on molecular weight to get the desired bands. Wells were filled with 5 µl DNA mixed with 2–3 µl loading dye. After completing the electrophoresis process, the gel was stained and visualized under UV light. DNA bands were separated from the gel using a sterilized pipette tip and mixed in PCR master mix as discussed in Table 4. Again, it amplified using a thermal cycler and the same conditions

mentioned in Table 5. The final product was confirmed through Gel electrophoresis.

Gel elelctropherisis for PCR product

1% agarose gel was prepared as mentioned above.

Fragment length polymorphism (RFLP)

After PCR then all samples PCR products were digested overnight at 37 °C with restriction enzyme "**FseI**" that fragment the sequence as follows:

At C; 148 + 156 cut into two slices.

At G; 304

(NEB, UK) according to the manufacturer's protocol as given below;

- Enzyme = 1 µl
- Buffer = 5 µl
- DNA = 20 µl
- Nucleolus free water = 24 µl
- Total = 50 µl

Gel electrophoresis restriction reaction

2% gel was prepared for this step as mentioned above. Then the product was analyzed by agarose gel electrophoresis and visualized under UV illumination. The image was recorded and saved.

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None.

Author contributions
TS were responsible for conceptualization, methodology, software, formal analysis, writing—original draft, visualization. MAM, ZUN, NA were responsible for methodology, software, supervision, funding acquisition, writing—review & editing. RA, AK, and NK were responsible for software, formal analysis, writing—review & editing. The authors read and approved the final manuscript.

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Availability of data and materials
Data will not be shared publicly. The datasets and materials used and/or analyzed during the current study can be made available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study protocol had the ethical approval of the Ethical Committee of Islamia University Bahawalpur, Pakistan. Informed written consent was taken from all the participants before inclusion in the study. All methods were carried out in accordance with Helsinki guidelines and regulations.

Consent for publication
Not applicable, as no individual or personal data were used in this study. All authors consent on publication of the submitted work.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

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