Screening of exon 2 of ARL6 gene in a consanguineous Bardet-Biedl Syndrome effected families from Khyber Pakhtunkhwa, Pakistan

By

Sumbal Wazir

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Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University Islamabad

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A dissertation submitted in partial fulfillment of the requirement for the degree of Master of Philosophy

In

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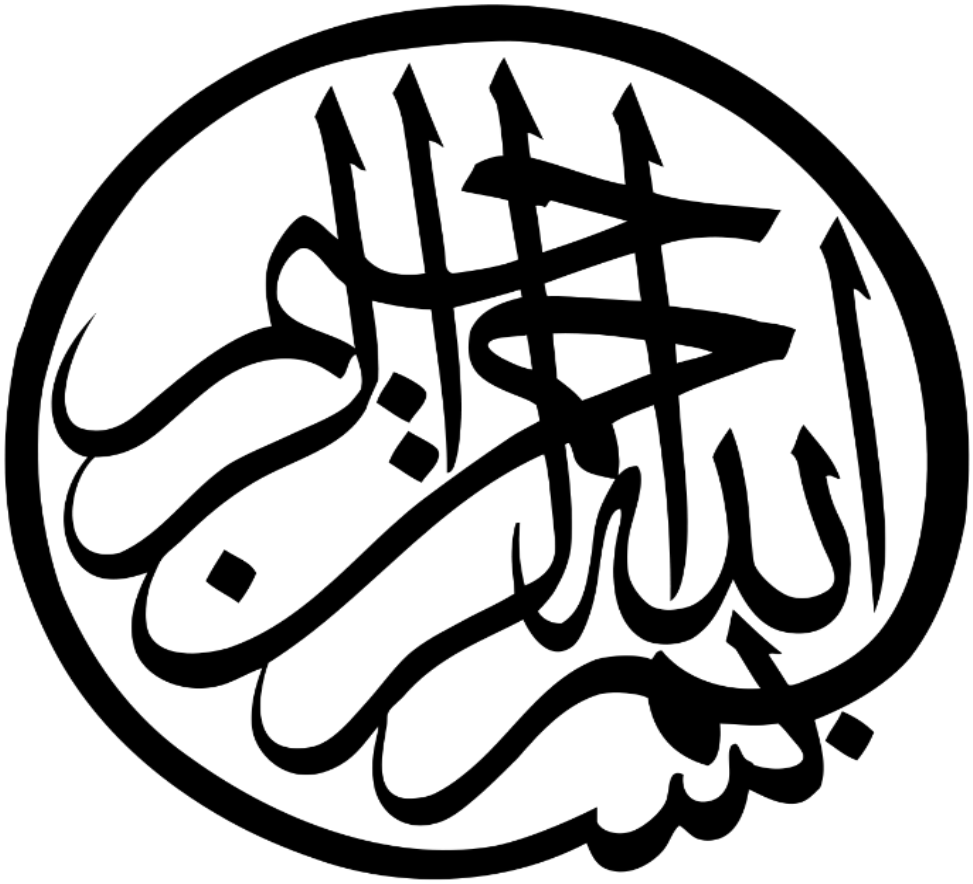
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CERTIFICATE

This dissertation "**SCREENING OF EXON 2 OF ARL6 GENE IN A CONSANGUINEOUS BARDET-BIEDL SYNDROME EFFECTED FAMILIES FROM KHYBER PAKHTUNKHWA, PAKISTAN**" submitted by **Ms. Sumbal Wazir** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Molecular Biology.

Supervisor: _____

Dr. Sabika Firasat

External Examiner: _____

_____

Dr. Amina Zuberi

Chairperson

Date: _____

DECLARATION

I **Sumbal Wazir**, student of **M. Phil. Molecular Biology**, Session **2021-2023**, hereby declare that the material and information contained in this thesis titled "**SCREENING OF EXON 2 OF ARL6 GENE IN A CONSANGUINEOUS BARDET-BIEDL SYNDROME EFFECTED FAMILIES FROM KHYBER PAKHTUNKHWA, PAKISTAN"** is my own work and has not been printed, published or submitted as research work, thesis or publication in any University or Research Institute in Pakistan or abroad.

_____

Sumbal Wazir

DEDICATION

To my father, Muhammad Naeem Khan wazir, who has always told me to go and live my dream.

My mother who supported me in my every decision, my three incredible sisters, and my brothers.

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Last, to the youngest one in the family, Muhammad Uzair Wazir.

SUMBAL WAZIR

List of Abbreviations

BBS	Bardet-Biedl Syndrome
PD	Polydactyly
RCD	Rod/Cone Dystrophy
RP	Retinitis Pigmentosa
ERG	Electroretinography
OS	Outer Segment
IFT	Intraflagellar Transport
IS	Inner Segment
Shh	Sonic Hedgehog
mTOR	Mammalian Target of Rapamycin
GPCR	G-protein Coupled Receptors
PC	Polycystin
ARF	ADP-ribosylation Factor
ACY1	Aminoacylase-1
CFAP	Cilia and Flagella Associated Protein
SCLT1	Sodium Channel and Clathrin Linker 1
SCAPER	S-phase cyclin A associated protein in ER
SCAPER	S-phase cyclin A associated protein in ER
SNP	Single Nucleotide Polymorphism
NGS	Next Generation Sequencing
LCA	Leber Congenital Amaurosis
PCR	Polymerase Chain Reaction

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ABSTRACT

Laurence and Moon originally recognized the ciliopathy known as Bardet-Biedl (BBS) condition in 1866. By its primary and secondary characteristics, BBS syndrome can be identified. The main signs of BBS are rod/cone dystrophy (RCD), polydactyly (PD), obesity, gene defects, kidney abnormalities, and learning difficulties. Common BBS secondary symptoms include developmental delay, dental problems, heart defects, speech difficulties, syndactyly or brachydactyly, poor coordination, olfactory anomalies, diabetes mellitus, hypertension, liver disorders, and craniofacial dismorphism. Most people with polydactyly at birth appear healthy, but later in life they are diagnosed with BBS. The symptoms of BBS appear in relation to age; in early age only a few symptoms are obvious; other symptoms evolve after first decade of life. The prevalence of BBS varies in isolated, inbred, and consanguineous communities, where it is estimated to affect 1 in 150 000 persons worldwide. Numerous studies have that about 26 genes are implicated in the condition. Initial gene finding investigations for BBS syndrome showed autosomal recessive mode of inheritance, but subsequent study complicated the genetics of BBS and prevailed incomplete penetrance and triallelic inheritance. The most frequently altered genes in Pakistan and India are BBS10 and BBS3/ARL6. The purpose of the current studies was to study clinical characteristics of BBS cases and to identify the molecular causes of familial cases of Bardet-Biedl syndrome in local population. Ethical approval for this study was obtained from Bio-Ethical Committee of Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, Hayatabad Medical Complex and Khyber Teaching Hospital, Peshawar, Pakistan. Ophthalmologists identified Retinitis Pigmentosa (RP) in all of the participating families, and following thorough interviewing, BBS families were selected. Blood samples and clinical records were collected from affected and unaffected members. For molecular genetic analysis, the genomic DNA was isolated. For the purpose of analyzing mutations, exon 2 of the ARL6 gene was amplified using primers. The amplified products were purified and sent for Sanger's sequencing. Sequence analysis did not show any polymorphism or mutation in the exon 2 of ARL6 gene in BBS identified families. To find a molecular genetic basis of disease, the remaining exons of ARL6 and other genes should be screened. The findings of this study also indicated that consanguinity is a factor in our population's high incidence that are recessively inherited, including BBS. Due of this, all participating families were provided with genetic counseling.

CHAPTER 1.
INTRODUCTION

1. INTRODUCTION

1.1. Human eye

The eye is a unique organ, from an anatomical and physiological point of view, since it has multiple very distinct structures with specialized physiological roles (John and Maria, 2019). The eye is one of the most complex organs of the human body. In the human eye, three layers can be distinguished. The outer region consists of the cornea and the sclera. The middle layer of the eye is composed of the iris, the ciliary body and the choroid. The inner layer of the eye is the retina, a complex, layered structure of neurons that capture and process light. The three transparent structures surrounded by the ocular layers are called the aqueous, the vitreous and the lens. The normal human eye (Figure 1) measures approximately 22 to 27 mm in anteroposterior diameter and 69 to 85 mm in circumference. The human eyeball consists of three primary layers, with each of those three layers being sub dividable (Barry D. Kels MD, JD *et al*., 2015).

1.1.1. Human Retina:

The retina is the tissue that lines the inner surface of the eye, surrounding the vitreous cavity. During embryogenesis, the vertebral retina develops from the optic cup. The latter is formed by invagination of the optic vesicle, which is an outgrowth of the embryonic forebrain. The inner wall of the optic cup (surrounding the vitreous cavity) ultimately becomes the neural retina; the outer wall (surrounded by the choroid and sclera) becomes the retinal pigment epithelium (RPE) (Schubert. 2009).

Microglia, astrocytes, and Müller cells, two forms of microglia, make up the three different types of glial cells that make up the human retina. In order for photoreceptors and neurons to function properly, microglia provide homeostatic and metabolic support. However, they do contribute to the structural stability of the foveal tissue and enhance the light transmission through the tissue to the photoreceptors. Müller cells of the foveolar do not promote the activity of photoreceptors and neurons. (Reichenbach, A., & Bringmann, A. 2020).

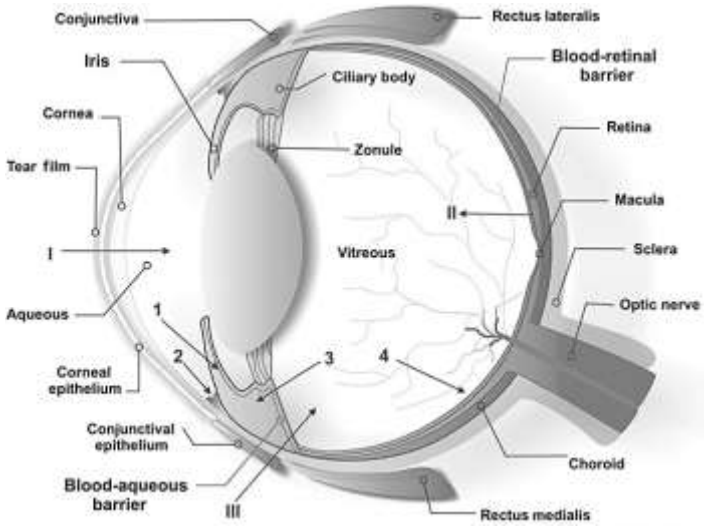

Figure 1.1 Schematic illustration of the structure of eye (Barar et al., 2009)

1.2. Eye Diseases:

Most individuals fear losing their ability to see, which is sometimes regarded as the most significant of all the senses. It is frequently believed that the main sense that allows a person to work is vision and carry out separate tasks. As the population ages and the prevalence of blinding ocular disorders rises, significant efforts are still being made to prevent vision loss.

Visual impairment is a major burden in every country, according to all measures of quality of life, including disability-adjusted life years (Chiang *et al*., 2006). The most common causes of irreversible blindness and visual impairment are disorders affecting the posterior region (Pascolini and Mariotti, 2012), which include diabetic retinopathy (DR), glaucoma, age-related macular degeneration (AMD), macular oedema owing to retinal vein occlusion (RVO), posterior uveitis, cytomegalovirus (CMV) retinitis, and retinitis pigmentosa (RP) (Waite *et al*., 2017).

1.2.1. Retinitis Pigmentosa:

Retinitis pigmentosa (RP) is a group of hereditary diseases causing photoreceptor degeneration (Hartong *et al*., 2006 and Hamel, 2006). 1 in 4000 persons have RP, which is a primary cause of blindness (Bunker et al, 1984., Grøndahl., 1987). Retinitis pigmentosa is a condition that affects roughly 1 in 4000 people globally. Some populations might be underestimating the prevalence. In the Jerusalem region, the prevalence of nonsyndromic retinitis pigmentosa is 1:2086, which is around 2.5 times greater than the prevalence in populations in North America and Europe. (Sharon and Banin, 2015).

1.2.2. Types of Retinitis Pigmentosa:

RP is classified as nonsyndromic (not affecting other organs or tissues); syndromic (affecting other neurosensory systems such as hearing); or systemic (affecting multiple tissues) (Abigail *et al.,* 2023).

1.2.3. Syndromic RP:

Syndromic RP is frequently caused by mutations in ciliary function-related genes, but this is not always the case. Usher syndrome, which manifests with a varied degree of neurosensory hearing loss, is arguably the most prevalent ciliopathy (Boughman *et al*., 1983). Bardet-Biedl syndrome is another prominent RP syndromic variant (Mockel *et al*., 2011). Systemic metabolic and mitochondrial problems are also linked to syndromic RP. However, some extra-ocular characteristics of syndromic RP, such as polydactyly, can be surgically corrected at a young age. Extra-ocular characteristics of syndromic RP can be extremely subtle (for instance, a diminished sense of smell) or easily overlooked by the examining ophthalmologist. As a result, gathering a careful, comprehensive history that covers these diverse extra-ocular anomalies is crucial for making a diagnosis (DeLuca *et al*., 2016).

1.2.4. Non Syndromic RP:

There are two forms of non-syndromic RP.

a) Typical form: RP is a chronic condition that often progresses over many years. However, there are rare instances with a sluggish progression that never results in blindness or a quick evolution over a period of twenty years. Three stages make a concise breakdown of the disease progression.

i) early stage ii) mid stage and iii) end stage.

b) Clinical Form: Non-syndromic RP comes in a variety of forms that can be categorized according to a number of factors. For examples, Age of onset, fundus appearance, mode of inheritance (Hamel., 2006).

1.3. Ciliopathies:

Ciliopathies are diseases caused by the dysfunction of motile and non-motile primary cilium (Strong *et al*., 2021). Numerous cellular activities involve primary cilia. Such include stem cell maintenance, regulation of the cell cycle, development, migration, polarity, differentiation, and transduction of stimuli (Álvarez-Satta., 2017). One ciliary gene is known to contribute to the emergence of two or more separate diseases, such as MKKS, which is connected to Bardet-Biedl syndrome (BBS, MIM #209900) or McKusick-Kauffman syndrome (MKKS, MIM #236700). The clinical and molecular diagnosis are both made more difficult by this genetic heterogeneity, as well as the substantial phenotypic variability, overlapping abnormalities, and the progressive emergence of numerous traits during infancy and adolescence (Bachmann-Gagescu., 2014). High clinical and molecular heterogeneity, as well as significant clinical overlap between entities, are characteristics of ciliopathies (Zaki *et al*., 2011). Hydrocephalus, infertility, chronic respiratory conditions, congenital heart problems, and organ lateralization disorders are all brought on by motile cilia failure (Reiter *et al*., 2017). Retinal dystrophy, anosmia, hearing loss, central obesity, polydactyly, rib cage abnormalities, hypogonadism, genital abnormalities, ataxia, epilepsy, mental disability, brain malformations, facial abnormalities, renal abnormalities, and liver diseases are all caused by non-motile cilia dysfunction (Reiter, 2017).

1.4. Bardet-Biedl Syndrome (BBS):

An autosomal recessive systemic disorder called Bardet-Biedl syndrome (OMIM: 209900; BBS: Online Mendelian Inheritance in Man) is characterized by retinitis Pigmentosa, polydactyly, obesity, cognitive deficits, renal abnormalities, and hypogonadism (Forsythe et al., 2018). It is a rare genetic illness called Bardet-Biedl syndrome (BBS) causes severe multiorgan dysfunction (Forsythe and Beales, 2013). BBS can be divided into 22 types at the molecular level according to the recent discovery of BBS22 (also known as IFT74), a gene that causes BBS (Mardy *et al*., 2021).

1.4.1. BBS historical overview:

Doctors Moon and Laurence published their research on a family with retinitis Pigmentosa, obesity, and intellectual impairment in the 1880s, when they first described BBS (Savin, 1935). Doctors Bardet and Biedl independently described families with obesity, polydactyly, and retinitis pigmentosa in 1920 and 1922 (Forsythe *et al*., 2013). Laurence-Moon-Bardet-Biedl syndrome became the name of the condition in 1925. Later, it was proposed that the syndrome could be divided into two distinct conditions, Laurence-Moon syndrome and Bardet-Biedl syndrome, depending on whether or not a patient had spastic paraparesis. This was based on historical descriptions of patients with the disease. The allelic nature of the syndromes is now well acknowledged, and the infrequent occurrence of spastic paraparesis as a sign of the syndrome's diversity (Forsythe *et al*., 2018). The condition is now known simply as Bardet-Biedl syndrome (Forsythe *et al*., 2013).

1.4.2. Bardet-Biedl Syndrome incidence rate:

In populations that are inbred or consanguineous, BBS is very common. BBS has a prevalence of 0.7/100,000 in the general population and a newborn prevalence of 0.5/100,000 (Prevalence and Incidence of Rare Diseases: Bibliographic Data. Orphanet Report Series Number 1 January 2020). The prevalence of the disease varies between isolated, inbred (Bedouin and Newfoundland - 1:13,500 and 1:16,000 (Water and Beales, 2011), consanguineous (Arab - 1:65,000) (Riise *et al*., 1997) and other populations (North America and Europe - 1:140,000 and 1:160,000) (Katsanis *et al*., 2001). Some isolated human communities are characterized by unusually high occurrence of this disease (Sheffield, 2004). For example, 13 BBS patients were registered among 48,000 inhabitants of the Faroe Islands, leading to disease frequency estimates of 1:3,700 (Hjortshøj *et al*., 2009). BBS prevalence in Newfoundland was reported to approach 1:18,000 (Moore *et al*., 2005). These variations in prevalence are the result of a variety of factors, including consanguinity, which is a societal practice in countries like Pakistan, Iran, Kuwait, and the Middle East. Even Ashkenazi Jews, who appear to be the most genetically investigated pioneer population, have not yet been made aware of BBS, as there is very little global comprehensive epidemiological data surrounding this disease. Few BBS instances have been reported to date in Asia, South America, Africa, and Eastern Europe; a comprehensive investigation of these populations is still needed (Suspitsin and myanitov, 2016; Khan *et al*., 2016).

1.4.3. Biology of the disease:

Most vertebral cells have cilia, which are highly preserved cellular features, protruding from their apical surfaces. They can be categorized as either motile or immotile (primary) cilia (Baker and Beales, 2009). Motile cilia are arranged in a '9 + 2' microtubule arrangement, where nine microtubule pairs surround a center doublet. These cilia cause liquids to flow or move. Primary ciliary dyskinesias caused by defective motile cilia can cause bronchiectasis, infertility, and left-right asymmetry (Waters and Beales, 2011). Immotile (primary) cilia have a "9+0" arrangement, identical in structure to the motile cilia but lacking the central pair, and are assumed to primarily serve as a sensory organelle regulating the signal transduction pathways (Baker and Beales, 2009; Waters and Beales, 2011). Retinitis pigmentosa, polydactyly, situs inversus, learning disabilities, and cystic kidney, liver, and pancreas are clinical signs of immotile cilia defects (Gardes *et al*., 2009).

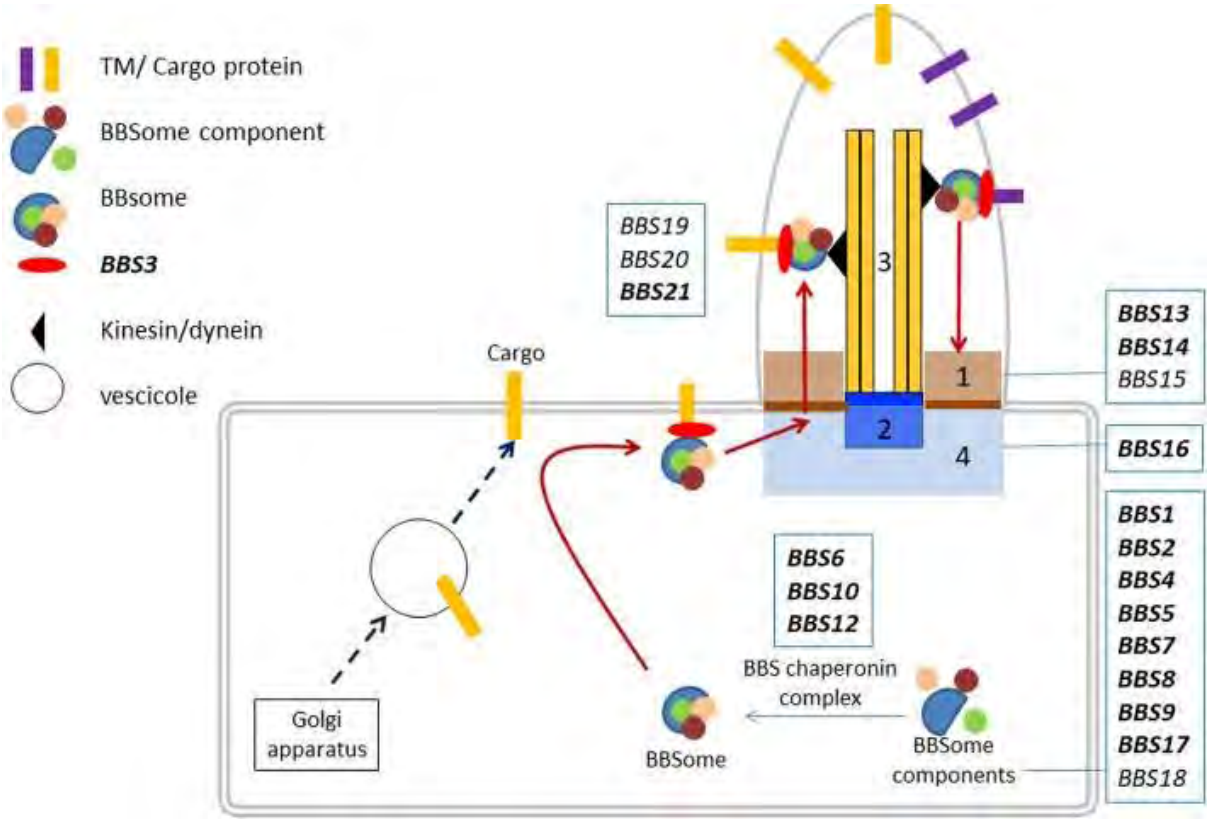

Figure 1.2 A major role of the BBSome relates to the trafficking of cargos to and from cilia including G protein–coupled receptors through a process that involves BBS3 (Zhao et al.,2022)

1.4.4. Features of BBS:

1.4.4.1. Major Features of BBS:

Retinal dystrophy: One of the most important features of BBS is the rod-cone dystrophy, affecting 94–100% of individuals (Niederlova., 2019). The patients may exhibit night blindness, loss of peripheral vision, a reduction in color perception, and a general loss of visual acuity (Weihbrecht *et al*, 2017). The modified ciliated cells known as rod and cone photoreceptor cells are in charge of both day and night vision. Rhodopsin is the primary protein of rod cells. The BBSome complex permits the transfer of this protein from the interior to the outside of rod cells. Rhodopsin accumulates and is mislocalized in rod cells as a result of mutations in BBSome genes. This causes the degeneration of the photoreceptors and disturbs the cellular homeostasis (Mockel, 2011).

Obesity is a common symptom (89%), it affects the thorax and belly, and it starts developing at a young age (between 2 and 3 years) (Niederlova, 2017). Our lab has studied the molecular pathophysiology of obesity in BBS and demonstrated that BBS proteins are critical for leptin receptor signaling in the hypothalamus (Seo *et al*., 2009). Obesity in BBS patients is caused by the loss of BBS proteins, which interfere with leptin receptor trafficking and cause hyperphagia and lower energy expenditure (Guo *et al*., 2016, 2019).

Polydactyl: Up to 80% of BBS patients may have postaxial polydactyly According to genotype-phenotype studies, BBS patients with BBS2 gene mutations had a greater penetrance of polydactyly. (Niederlova *et al*., 2019). Mesoaxial polydactyly has been linked to the gene BBS17, a negative regulator of ciliary trafficking mediated by BBSome and the Shh (Sonic hedgehog) signaling (Schaefer *et al*., 2014).

Renal impairment: Kidney disease is present in 52% of BBS patients. The advancement of chronic renal impairment is aided by hypertension and type 2 diabetes mellitus, which are also the leading causes of morbidity and mortality in BBS patients (Forsythe *et al*., 2017; Imhoff et al., 2011). The increased prevalence of renal abnormalities in patients with mutations in BBS2, BBS7, or BBS9 provides additional evidence that the BBSome is involved in renal development (Niederlova *et al*., 2019). Chronic kidney disease (CKD) is the major effect, which raises the morbidity and mortality rates in BBS patients. Most cases of renal illness are fully recognized by age five, but certain symptoms may be seen as early as the first year of life (Forsythe *et al*., 2016).

Intellectual disability: In 66% of BBS cases, cognitive impairment is evident .Either an intellectual handicap or deficiencies in linguistic fluency, attention span, faulty thinking, and emotional immaturity can be present in a patient. There is no known relationship between BBS proteins and cognitive dysfunction (Niederlova *et al*., 2019). However, we recently showed that context fear conditioning, a type of associative learning, is significantly impaired in BBS mice. The information shows that decreased hippocampus neurogenesis is the cause of the behavioral deficits seen in BBS animals (Pak *et al*., 2021).

Reproductive abnormalities: Hypogonadism and genitourinary malformations have an incidence of 59% (Niederlova., 2019) but it may not be detected until patients present with delayed puberty (Deveault *et al*., 2011; Mujahid *et al*., 2018). Male abnormalities can range from hypogonadotropic hypogonadism to small-volume testes, cryptorchidism, and micropenis (Mujahid *et al*., 2018). Uterine, fallopian, ovarian, or vaginal hypoplasia or atresia are examples of female anatomic abnormalities (Niederlova, 2019).

1.4.4.2. Minor Features of BBS:

About 47–100% of BBS patients have olfactory impairment, which includes anosmia and hyposmia (Braun *et al*., 2014; Tadenev *et al*., 2011). While olfactory cilia abnormalities are assumed to be the cause, people with BBS have an abnormal-looking olfactory bulb on brain MRIs (Braun *et al*., 2016).

Hirschsprung disease, inflammatory bowel disease, and celiac disease are some other gastrointestinal anomalies (unpublished data from the Clinical Registry Investigating Bardet-Biedl Syndrome, CRIBBS). Of BBS patients, up to 30% may have heterotaxy (Olson *et al*., 2019). Metabolic syndrome, type 2 diabetes, polycystic ovarian syndrome, and hypothyroidism are all frequent endocrinopathies (Mujahid *et al*., 2018).

Dental crowding, short roots and taurodontism, posterior crossbite, enamel hypoplasia, high-arched palate, and hypodontia or microdontia are a few examples of primary malformations. Poor oral hygiene, periodontal disease, dental caries, drug-induced gingival hyperplasia, xerostomia, changed taste, and speech impairments are examples of potential secondary oral problems (Panny *et al*., 2017).

Liver disease comprises periportal fibrosis, non-alcoholic fatty liver disease (NAFLD), anomalies of the bile duct with cystic dilatation, and other conditions (Branfield Day *et al*., 2016).

Table 1.1.: Clinical Signs of BBS Patients (Melluso et al., 2023)

Primary Diagnostic Features	Secondary Diagnostic Features	Described BBS Features Non Included in the Diagnostic Criteria
Retinal Degeneration	Strabismus, cataracts, and astigmatism	Cutaneous Dermatoses
Obesity	Metabolic/endocrine abnormalities (metabolic syndrome, subclinicalhypothyroidism, polycystic ovary s.)	Hearing loss
Postaxial polydactyly	Brachydactyly/syndactyly	Asthma
Renal Anomalies	Anosmia/olfactory dysfunction	Dysregulated immune andhematopoietic systems
Learning Disabilities	Neurodevelopmental abnormalities (developmental delay, speech delay, epilepsy, behavioral disturbances, ataxia/poor coordination, mild spasticity)	Musculoskeletal abnormalities
Hypogonadism and Genitourinary Abnormalities	Liver and other gastrointestinal diseases (Hirschsprung disease, inflammatorybowel disease, celiac disease)	
	Cardiovascular and thoraco-abdominal abnormalities	

1.5. Diagnosis of BBS:

According to the diagnostic criteria outlined by Beales *et al*., Bardet-Biedl syndrome is classified as a pleiotropic disorder if at least four main features, or three major features and at least two minor features, are present (Beales *et al*., 1999).

The age at which patients are diagnosed varies greatly depending on when their symptoms of rod-cone dystrophy first appear. Although it can start in childhood, it is more frequently diagnosed between the ages of 5 and 10 and usually begins with night blindness (Beales *et al*., 1999).

Obesity in children or isolated polydactyly at birth typically do not require referral. Typically, siblings of affected children are diagnosed earlier. Without a family history, antenatal diagnosis is extremely rare, but BBS may be suspected based on the ultrasound findings of echogenic kidneys and polydactyly.

Although there is inadequate evidence to support it, children who appear with renal abnormalities or renal failure may have their diagnoses made earlier than those who do not. A small percentage of people have isolated rod-cone dystrophy without other BBS-related symptoms, and they are frequently diagnosed as adults. Due to the development of panel-based genetic testing and significant diagnostic initiatives like the UK 100,000 genomes project, these people are now being identified (Samuel and Farsides, 2017).

Currently, the preferred diagnosis tool is a gene panel. The use of whole exome sequencing (WES) and whole genome sequencing (WGS) may improve coverage, assist in the identification of non-coding variations, and facilitate the discovery of new genes. However, a drawback of increasingly powerful diagnostic sequencing techniques is the discovery of pathogenic variants in non-BBS genes and variations of uncertain significance (VUS) in BBS genes, in addition to greater cost (Vears *et al*., 2017).

When individuals only exhibit one or two key non-specific diagnostic criteria, such as obesity and/or learning impairments, this might create a diagnostic conundrum. Patients must carefully consent to the use of WES and WGS, and a plan must be in place to cope with VUS and unexpected results (Mitchison and Valente, 2017).

1.6. Genes involved in BBS:

BBS has been linked to 21 BBS loci (BBS1-C8or f37/BBS21), who's mutations would account for about 80.0% of patients with the condition (Weihbrecht *et al*., 2017; Manara *et al*., 2019). As a result of advancements in genetics, the syndrome is now linked to a total of twenty-six genes, and this number has been steadily growing over the years (Meyer *et al*., 2022). Around 70-80% of cases worldwide can be attributed to mutations in BBS1 to BBS18 (M'hamdi O *et al*., 2014). In Western countries, approximately half of the diagnoses can be traced back to mutations in three specific genes: BBS1, BBS2, and BBS10 (Forsyth *et al*., 2020).

In Northern Europe and North America, the majority of genotypes (51 and 20%, respectively) are caused by mutations in BBS1 and BBS10 (Forsythe *et al*., 2013). The classic example of a gene that causes many ciliopathies in addition to BBS is CEP290, which also causes Joubert syndrome, Leber congenital amaurosis, Meckel syndrome, and Senior-Loken syndrome (Mitchison and Valente, 2017). List of genes responsible for BBS syndrome are mentioned in table 1.2.

Table 1.2.: Known Causative Genes of Human Bardet–Biedl Syndrome (Melluso et al., 2023)

Gene	Name	Chromosomal Coordinate	Localizationof the Protein in the Cell	Tissue Specificity	Protein Function
BBS1	Bardet-Biedl syndrome 1	11q13.2	Cilium andbasal body	Low	Component of BBSome complex
BBS2	Bardet-Biedl syndrome 2	16q13	Cilium andbasal body	Low	Component of BBSome complex
BBS3/ARL6	Bardet-Biedl syndrome 3/ADP ribosylation factor like GTPase 6	3q11.2	Cilium, basalbody, transition zone and cytosol	Low	GTP-binding protein involved inciliary trafficking[146]
BBS4	Bardet-Biedl syndrome 4	15q24.1	Cilium andbasal body	Low	Component of BBSome complex
BBS5	Bardet-Biedl syndrome 5	2q31.1	Basal body	Low	Component of BBSome complex
BBS6/MKKS	Bardet-Biedl syndrome 6/MKKS centrosomal shuttling protein	20p12.2	Cilium andbasal body	Low	Chaperonin like protein assistingBBSome formation

BBS7	Bardet-Biedl syndrome 7	4q27	Cilium andbasal body	Low	Component of BBSome complex
BBS8/TTC8	Bardet-Biedl syndrome 8/tetratricopeptide repeat domain 8	14q31.3	Cilium, IFT and basal body	Low	Component of BBSome complex
BBS9	Bardet-Biedl syndrome 9	7p14.3	Cilium	Low	Component of BBSome complex
BBS10	Bardet-Biedl syndrome 10	12q21.2	Basal body	Low	Chaperonin like protein assistingBBSome formation
BBS11/TRIM32	Bardet- Biedl syndrome 11- tripartite motif containing 32	9q33.1	Intermediate filaments	Low	E3 ubiquitin ligase; it promotes degradation of several targets[147]
BBS12	Bardet-Biedl syndrome 12	4q27	Basal body	Low	Chaperonin like protein assistingBBSome formation
BBS13/MKS1	Bardet-Biedl syndrome 13/MKS transition zone complex subunit 1	17q22	Basal body	Low	Component of the tectonic-like complex localized at the transitionzone of primary cilium[148]
BBS14/CEP290	Bardet-Biedl syndrome 14/centrosomal protein 290	12q21.32	Basal body and centrosome	Low	Centrosomal protein involved inprimary cilium formation[149]
BBS15/WDPCP	Bardet-Biedl syndrome 15/WD repeat containing planar cell polarity effector	2p15	Cytosol, axoneme andplasma membrane,	Low	Controls ciliogenesis[150]
BBS16/SDCCAG8	Bardet-Biedl syndrome 16/SHH signaling and ciliogenesis regulator SDCCAG8	1q43-q44	Basal body, transition zone and centriole	Low	Involved in ciliogenesis and SonicHedgehog signaling pathway
BBS17/LZTFL1	Bardet-Biedl syndrome 17/leucine zipper transcription factor like 1	3p21.31	Cilium andbasal body	Mainly in lymphoid tissue	Regulator of BBSome trafficking andSonic Hedgehog signalling[151]
BBS18/BBIP1	Bardet-Biedl syndrome 18/BBSome interacting protein 1	10q25.2	Cytosol	Mainly in testis	Component of BBSome complex
BBS19/IFT27	Bardet-Biedl syndrome 19/intraflagellar transport 27	22q12.3	Cilium, IFT and basal body	Low	Intraflagellar trafficking (IFT-B)component[151]
BBS20/IFT172	Bardet-Biedl syndrome 20/intraflagellar transport 172	2p23.3	Vesicles	Low	Intraflagellar trafficking (IFT-B)component[152]

BBS21/ CFAP418/ C8orf37	Bardet-Biedl syndrome 21/ cilia and flagella associated protein 418	8q22.1	Basal body and ciliary root	Low	Unknown[153]
BBS22/IFT 74	Bardet-Biedl syndrome 22/intraflagellar transport 74	9p21.2	Cilium, IFT and basal body	Low	Intraflagellar trafficking (IFT-B)component[152]
CEP19	Centrosomal protein 19	3q29	Centrosome	Low	Recruits the RABL2B GTPase to theciliary base and intraflagellar transport (IFT) complex B[154]
NPHP1	Nephrocystin 1	2q13	Transitionzone	Mainly in skeletal muscle	Cell-matrix signaling at focaladhesions[155]
SCAPER	S-phase cyclin A associated protein in the ER	15q24.3	Endoplasmic reticulum andciliary tip	Low	Ciliary dynamics and disassembly[156]
SCLT1	Sodium channel and clathrin linker 1	4q28.2	Centriole	Low	Component of distal appendageswhich anchor the cilium to the plasma membrane, involved in ciliogenesis[157]

1.6.1. ARL6 (MIM 608845) and its role in BBS:

ARL6 (MIM 608845), a gene that causes BBS3, is found on chromosome 3's short arm (3p12-p13). It is known as ADP-ribosylation factor-like protein 6 and it encodes a cytosolic protein that is 186 amino acids long (Ingley *et al*., 1999). Nine exons make up the ARL6 gene. ARL6 has conserved elements from the RAS superfamily's ARF family, including as an N-terminal myristoylation site, a hydrophobic alpha helix, and a GTP-binding site. It has been suggested that ADP-ribosylation factors (ARFs) are crucial for intracellular membrane trafficking (Chiang *et al*., 2004). ARLs have undergone significant evolutionary conservation, and the majority of them have the consensus sequences needed for GTP binding and hydrolysis (Pasqualato *et al*., 2002). First discovered in a J2E erythro-leukemic cell line, ARL6. The ARL6 mRNA is increased during the differentiation of erythroid cells brought on by erythropoietin and downregulated during the differentiation of macrophages brought on by interleukin-6, suggesting a potential role in hemopoietic development. ARL6 exhibits a tissue-specific expression pattern in adult mice, with the highest expression seen in the brain and kidney (Ingley *et al*., 1999). BBS3 is assumed to be caused by ciliary malfunction, like other

BBSs, because loss-of-function mutations of ARL6 in C. elegans affect cilia shape and function. ARL6's role in BBS3 is well supported by the observations that it is preferentially expressed in ciliated cells, including sensory neurons, and implicated in intraflagellar transport in C. elegans (Takada *et al*., 2005). BBS syndrome is caused by 16 known mutations in the gene BBS3/ARL6, including splice site, missense, nonsense, and deletion mutations (Sathya Priya *et al*., 2015).

Among Saudi BBS patients, there is an unusual predominance of ARL6/ARL6 gene mutations, all of which are novel, in striking contrast to the BBS reports from Caucasians in which ARL6 gene mutations are extremely rare (Abu Safieh *et al*., 2010). In Indian population, ARL6 gene accounted for 18 per cent of the mutations, as against worldwide reports (0.4%) (Sathya Priya *et al*., 2015).

1.7. The BBSome function and structure:

Eight proteins from the genes BBS1, BBS2, BBS4, BBS5, BBS7, TTC8, BBS9, and BBIP1 make up the multisubunit complex known as BBSome (Khan *et al*., 2016). The BBSome goes through a stepwise assembly process (Zhang *et al*., 2012). In terms of structure, BBS2 and BBS7 come together to make up the upper section of the BBSome, while BBS5, BBS8, and BBS9 constitute the lower part of the complex. A corkscrew-shaped arrangement comprising BBS1, BBS4, and BBS18 serves as the connecting link between the upper and lower sections (Chou *et al*., 2019). BBSome assembly relies significantly on three BBS proteins that resemble chaperonins, namely BBS6, BBS10, and BBS12 (Seo *et al*., 2010). The main role of the BBSome is to act as an intermediary for intraflagellar transport (IFT) complexes. It broadens the types of cargo that the IFT machinery can handle and aids in the recruitment of cargo for export from cilia through retrograde transport (Liu and Lechtreck, 2018). To attract membrane cargo, the BBSome creates a membrane coat with the aid of a small-GTPase called ARL6 (also known as BBS3 and will be referred to as ARL6/BBS3 from now on). This entity is denoted as BBS3, and going forward, it will be referred to as ARL6/BBS3. The arrangement of the BBSome has been established through cryo-electron microscopy (Chou *et al*., 2019). The BBSome primarily exists in a closed and self-inhibited state when in solution, preventing it from binding to ARL6/BBS3. To form a connection with ARL6/BBS3, the BBSome undergoes a conformational alteration induced by a steric rotation of BBS1. This rotation permits ARL6/BBS3 to fit snugly between BBS1 and BBS7, shifting the BBSome from its closed state to an open configuration, enabling interaction with cellular

membranes. Once ARL6/BBS3 is attached to the BBSome, the resulting complex adopts a curved surface that is well-suited for membrane interaction. This convex surface is composed of ARL6/BBS3 along with certain segments of BBS2, as well as the β-propeller domains of BBS7 and BBS9. The binding of ARL6/BBS3 enhances the presence of positive surface charges on the BBSome, making it easier for the BBSome to associate with the membrane (Yang *et al*., 2020). Analyzing the structure of the BBSome has provided valuable insights into how the BBSome becomes activated to capture cargo effectively (Garcia, 2020).

1.8. Mode of inheritance:

Inheritance of Bardet-Biedl syndrome (BBS) is typically autosomal recessive. Despite the fact that oligogenic inheritance of BBS has been proposed in certain families with members who have been shown to have the clinical symptoms of BBS and variations in two or more distinct BBS-associated genes (Manara *et al*., 2019), this mode of inheritance has been refuted in other studies (Abu-Safieh *et al*., 2012). Copy number and intronic variation detection, as well as more thorough phenotypic characterization of those with variants of unknown importance, are likely to add to the evidence supporting classic autosomal recessive inheritance in BBS. (Lindstrand *et al* 2016; Shamseldin *et al.,* 2020). The therapeutic importance of modifier gene variations in BBS is now up for discussion (Yıldız Bölükbaşı., *et al* 2018).

1.9. Current Knowledge on Patients' Survival and Clinical Management:

Currently, there is no specific treatment tailored for this syndrome. However, a noteworthy finding from Moore et al.'s 22-year prospective cohort study on Newfoundland's population revealed a median survival of 63 years for patients with the syndrome (Moore *et al*., 2005). According to O'Dea's findings, approximately 25% of patients passed away before reaching the age of 44, while only 2% of unaffected siblings experienced a similar fate. Among the deceased patients, 72% had renal issues, with chronic uremia accounting for 38% of the recorded causes of death (O'Dea *et al*., 1996). Specialists with expertise in specific diseases, such as nephrologists, dental specialists, endocrinologists, psychologists/psychiatrists, dietitians, ophthalmologists, gastroenterologists, neurologists, urologists, gynecologists, dermatologists, and others, address the primary clinical manifestations (Forsyth *et al*., 2020).

1.9. BBS Therapies:

1.9.1. Setmelanotide:

BBS is characterized by two prominent symptoms: an insatiable and pathological hunger, along with obesity (Forsythe *et al*., 2013). Most patients with BBS have symptoms of hyperphagia in the first years of life (Sherafat *et al*., 2013). The BBS genes play a crucial role in the transportation of the leptin receptor (LEPR), an essential element of the melanocortin pathway. In regular circumstances, leptin binds to the leptin receptor found on proopiomelanocortin (POMC) neurons. Subsequently, the protein encoded by PCSK1 cleaves POMC, resulting in the formation of α–melanocyte-stimulating hormone. This hormone then activates the melanocortin-4 receptor (MC4R), leading to a reduction in food intake (Yang *et al*., 2019). Setmelanotide, an eight-amino-acid cyclic peptide, selectively attaches to MC4R, serving as a replacement for melanocyte-stimulating hormone in neurons expressing MC4R Setmelanotide's distinctive mode of operation stimulates MC4R and has the ability to counteract the impacts of genetic deficiencies that may arise earlier in the pathway, particularly in individuals affected by uncommon genetic obesity disorders. During earlier phase 2 trials, administering setmelanotide resulted in decreased body weight and reduced appetite in individuals with obesity caused by POMC and LEPR deficiencies (Clement *et al*., 2018). The treatment was well-received by participants, and there were no significant clinical elevations in blood pressure or heart rate, unlike the observations made with initial-generation MC4R agonists (Greenfield *et al*., 2009). Nonetheless, it is yet to be established whether setmelanotide's activation of MC4R can effectively diminish hunger and enhance weight loss in individuals with syndromic types of obesity, which could be linked to diminished activity in the MC4R pathway, such as BBS (Haws *et al*., 2020).

1.9.2. Future therapies for BBS:

Significant progress has been made in the last ten years in the development of therapeutic approaches that may one day be used to treat people with BBS and similar ciliopathies. However, there is a special obstacle in creating genetic treatments for BBS due to the abundance of disease-causing genes and private mutations, which are those seen in just one family (Mujahid *et al*., 2014).

Gene therapy research is one of the key areas of BBS (Pomeroy *et al*., 2021).In animal models of BBS, retinal degeneration has been treated utilizing gene therapy employing a viral vector, which is often used to target a single organ like the eye (Seo *et al*., 2013). The eye is

simple to access, and patients often don't exhibit symptoms until mid- to late infancy. Tools for CRISPR/Cas9 and gene editing therapy may be of interest (Kenny *et al*., 2017). For nonsense mutations (premature termination codons), which account for about 12% of Bardet Biedl cases, read-through therapy may be performed (Kenny *et al*., 2017) and its effectiveness on different ciliopathies has already been evaluated in the preclinical level (Bukowy-Bieryllo *et al*., 2016).

Antisense oligonucleotides, snRNAs, and RNA interference are a few splicing-correcting techniques that can be used to target BBS mutations that affect splicing. Additionally, the potential therapeutic effects in fibroblasts with a BBS1 mutation have been established in vivo (Forsythe *et al*., 2018).

For obesity caused by BBS, new treatments are being developed. Pomeroy et al. noted that techniques to lengthen sleep could be helpful to reduce obesity. (Pomeroy *et al*., 2021).

CHAPTER.2
MATERIALS
AND
METHODOLOGY

2. MATERIALS AND METHODS

2.1. Ethics statement:

The present study was conducted after the clearance from Bio-Ethical review committees, of Quaid-i-Azam University, Hayatabad Medical Complex and Khyber Teaching Hospital Peshawar, Pakistan.

2.2. Selection and clinical evaluation of BBS families:

Bardet-Biedl Syndrome Patients were separated after conducting a long detailed interview from those patients that were clinically diagnosed with Retinitis Pigmentosa (RP). BBS patients and their close relatives from different parts of Khyber Pakhtunkhwa were included in this study.

2.3. Pedigrees of Families:

The genetic connection among family members was investigated through a comprehensive interview with the typical family head. The mode of inheritance was determined through pedigree analysis. HaploPainter 1.043 was used to illustrate the family pedigrees, with circles denoting females and squares representing males. Filled symbols indicated patients with Bardet-Biedl syndrome, while hollow symbols represented healthy individuals. Departed family members were marked with a diagonal line on the symbol. Cousin marriages were represented by two double lines between the symbols. Roman numerals were used to indicate different generations, and Arabic numerals were used to identify specific members within each generation.

2.4. Blood Samples:

Blood samples from patients were collected from Hayatabad Medical Complex, Khyber Teaching Hospital and Frontier Institute of Ophthalmology, Peshawar. All the patients were included in the study with their complete consent.

2.5. Inclusion Criteria:

- Families with clinically diagnosed RP and having positive family history were included in the study, but only patients who had symptoms of the BBS syndrome underwent molecular investigation.

2.6. Exclusion Criteria:

- The study excluded patients who have clinically diagnosed RP but no positive family history.
- Patients with eye disorders other than RP were excluded
- Additionally, patients who met the inclusion criteria but chose not to participate in the study were also not included.

2.7. Blood Sample Collection:

5ml blood was collection from patients and their first hand relative in EDTA tube. All the EDTA tubes were properly labelled. The label displayed the individual's name, parent's name, and a unique anonymous identification (LAI) number assigned to each person to ensure their differentiation from one another. To prevent blood clotting, tubes containing EDTA were thoroughly mixed after blood collection and subsequently stored at -4°C in a Dawlance refrigerator.

2.8. Lab Work at Quaid I Azam University Islamabad:

- Chemicals for DNA extraction.
- Extraction of DNA
- Electrophoresis by using Agarose Gel
- Nano drop
- Primer designing
- Primer dilutions
- Polymerase Chain Reaction (PCR)
- PCR product purification
- Sequencing

2.9. Genomic DNA extraction:

The Phenol-Chloroform Method, an organic extraction technique, was utilized to extract the genomic DNA. The protocol of genomic DNA extraction is given below:

A. Day 1:

- Blood samples were defrosted and allowed to thaw at room temperature prior to the red blood cell (RBC) lysis.
- 750 ul of the labeled person's blood sample and 750 l of Solution A were transferred into the eppendorf tubes with a 1.5 ml capacity. The tubes were labelled with the person's UAI.
- Next, this mixture consisting of blood and solution A was mixed and then the tubes were allowed to rest for 25- 30 min at room temperature
- After 30 minutes lysis, centrifugation was done in Beckman microfuge at for 15 minutes at 13000rmp.
- Following centrifugation, half of the supernatant was discarded (300 µl) in a suitable beaker filled with water and bleach to prevent contamination.
- The remaining amount of pellet was washed with 400µl of Solution A once more, thoroughly mixed, and centrifuged for 15 minutes at 13,000 rpm.
- After centrifugation almost all the supernatant was discarded and then pellet was washed again with 400ul Solution A and then centrifuged at 13000rpm for 15 minutes.
- After 4th washing and centrifugation all the supernatant was discarded and pellet was dissolved in 400ul Solution B, 6ul Proteinase k and 25µl of 20% SDS by shaking.
- These sample containing in eppendorf tubes were incubated at 37°C for overnight for protein digestion in WBCs pellet.

B. Second Day:

- Fresh solutions were prepared for day two (C+D).
- On the second day, 500 µl of solutions C and D that was made by combining both solutions equally (50:50) were added.
- Each sample was centrifuged for 15 minutes at 13,000 rpm.
- New eppendorf tubes were labeled.
- Two layers (top and bottom) were created as a result of centrifugation. The top layer was removed and moved to an eppendorf with new labels.
- 500µl of solution D was added to the separated layer, shake erratically, and centrifuge at 13000 rpm for 15 min. Again two were two layers created. The sample's top layer was picked and transferred to fresh, labeled Eppendorf tubes.

- The tips were cut before picking the layers.
- Furthermore, the sample was mixed with 500ml of cooled iso-propanol and 55 ml of chilled sodium acetate (3M, pH 5-6). The tubes were repeatedly turned over to precipitate genomic DNA.
- After that, the sample was centrifuged for 15 minutes at 13,000 rpm, and a pellet was observed as a result.
- The pellet was washed with 200 l of 70% ethanol and centrifuged for 10 minutes at 13,000 rpm after the supernatant was discarded.
- The pellet was allowed to dry in the vacuum concentrator while the Supernatant was once more discarded.
- Air dried DNA was placed in an eppendorf tube, and a suitable volume (80-200 l) of TE buffer (Tris-EDTA) was added. The tube was then incubated overnight at 37 °C.

C. Third Day:

- The caps of the eppendorf tubes were sealed using Parafilm strips.
- To avoid denaturing the extracted DNA, samples were sealed and then given a 1-hour heat shock in a water bath at 73 °C.
- DNA solutions were centrifuged and put in labeled autoclaved screw cap tubes after being placed at room temperature for five minutes in the tubes.
- Then, for qualitative and quantitative analysis, 4 l of the extracted genomic DNA from each sample was mixed with 4 l of bromophenol blue dye and run on a 1% agarose gel.
- The gel was subsequently put through further analysis with a UV trans illuminator (Gel-Doc system) from Biometra, Gottingen, Germany, and kept in a cryobox at -20 °C.

Table. 2.1.: Concentrations and Compositions of solutions used for DNA extraction:

Solution	Concentration and composition
Solution A	Solution A is known as lysis solution and it consist of 0.32 M sucrose, 10mM Tris (pH-7.5), 5mM MgCl and Triton X 100 (1% V/V).
Solution B	Solution B is made up of 400mM NaCl, 10mM Tris (pH 7.5), and 2mM EDTA (pH 8.0).
Solution C	In DNA extraction, solution C is known as phenol, which aids in the removal of lipids and non-polar proteins from the solution.
Solution D	Chloroform and isoamyl alcohol make up solution D in a 24:1 ratio.
Proteinase K	100mg/ml working concentration was used.
20%SDS	10 g Sodium dodecyl Sulphate in 50 mL water.
70% Ethanol	60ml distilled water was added 140ml absolute ethanol (molecular grade) to prepare 70% ethanol.
0.2 mM TE Buffer	1 mM EDTA, 10 mM Tris hydroxyl (methylamino) methane, (pH 8.0)

2.10. Protocol of Agarose Gel Electrophoresis (1%):

Gel Electrophoresis was done for the confirmation of DNA sample. The protocol of Agarose Gel Electrophoresis is given below.

- IX TBE (Tris-Boric Acid-EDTA) buffer was used to dissolve 0.5 grams of agarose powder in a conical flask.
- To make a 1X TBE buffer, 100 ml of 10X TBE was mixed with 900 ml of distilled water in a 1000 ml bottle.

- To make the 10X TBE buffer, 40 ml of 0.5M EDTA, 108 g of Tris, and 54 g of Boric acid were combined. The combination was then given a final volume of 1000 ml by adding distilled water. Next, the pH was set to 8.0.
- To obtain a clear solution, the mixture was microwaved (Dawlance) for 1-2 minutes. Before putting the flask in the microwave, aluminum foil was used to seal off the aperture.
- After that, it is left to cool for a few minutes at room temperature.
- Comb and casting tray were set up in a rack.
- The clear solution was poured into the casting tray, making sure there were no air bubbles there, and letting it polymerize (solidify) at room temperature for 30 to 40 minutes.
- When the gel had set, the comb was carefully removed, and the gel was then put in the gel tank (Cleaver Scientific Limited CS-3000V), which was filled with the running buffer IX TBE.
- The loading samples were made by mixing 3 l of the extracted DNA from each sample with 3 l of the loading dye, 6 X bromophenol blue.
- The gel electrophoresis apparatus was run at 120 volts for 25 minutes.
- Once running was complete, the gel was seen under UV light using the Gel Documentation System (Cleaver Scientific Limited).
- On the computer, the image was saved for later use.

Table 2.2.: Composition of agarose gel and other required chemicals.

S.No	Solutions	Compositions of Different Required Solutions
1	**Agarose Gel 1% In 50ml**	0.5g Agarose + remaning volume upto 50ml filled with 1X TBE buffer + 2ul Ethidium Bromide
2	**Agarose Gel 2% In 50ml**	1g Agarose + remaning volume upto 50ml filled with 1X TBE buffer + 5ul Ethidium Bromide
3	**10X TBE Buffer used as Gel Preparation Buffer**	EDTA (3.65g) + Tris (54g) + Boric Acid (27.5g) + Deionized water upto 500ml
4	**Ethidum Bromide(100ml)**	Ethidium Bromide (1g) + Auto claved filtered water upto 100ml
5	**1X TBE Buffer 1000ml (Gel Running Buffer)**	900ml distilled water + 100ml 10X TBE
6	**Bromophenol (Loading Dye 25ml)**	Auto claved filtered water up to 25ml Sucrose 10g + Bromo-phenol blue(0.0875)

2.11. Nanodrop:

The concentration and purity of the extracted DNA were measured on nanodrop (Thermo-Scientific 2000) using TE buffer as a blank and the DNA samples, respectively.

2.12. WizPrep gDNA Kit Method:

DNA is extracted from whole blood simply and more conveniently, in a short period of time, that is from half to a few hours, depending on sample size. There is fast and easy processing using a rapid spin-column format, with no use of phenol or chloroform. The protocol is given below.

- Take 200µl of whole blood, add 200 µl of GB Buffer and 20 µl of Proteinase K.
- Vortex the above mixture and incubate at 56°C for 20 minutes.
- Add 200µl of 100% Ethanol in above mixture after incubation.
- Vortex the tubes to mix properly.
- Transfer the lysate (above mixture) into column that is attached with a collecting tube.
- Give short spin to the column (1 minute at 13000 rpm).
- Discard the filtrate and transfer the column to new collection tube.
- Add 500µl of W1 Buffer in column and centrifuge for 1 minute at 13000 rpm.
- Discard the filtrate, add 500µl of W2 Buffer, and centrifuge it for 1 minute at 13000 rpm.
- Discard filtrate and shift the column to newly labelled Eppendorf tubes.
- Add 50-100 µl Elution Buffer and keep it at room temperature for 1 minute.
- Centrifuge it for 1 minute at 13000 rpm and discard the column.
- The eluted purified DNA is extracted.
- Store the extracted DNA at 20°C for a few days and at -70°C for long term storage.

2.13. Primer Designing For Selected Region Amplification:

The primers for the amplification of the specified DNA region (exon 2) of the BBS3/ARL6 gene were designed using Primer-3 software https://primer3.ut.ee/ to analyze mutations and polymorphisms. The general conditions including annealing temperature, size of the amplitude, the concentration of salt, and length of primer were selected for the optimum level. The Ensemble website: http://asia.ensemblr.org/Homo_sapiens/Info/Index provided the reference sequence that was essential for building primers. BLAT (Blast Like Alignment Tool)

on the UCSC genome browser and the In-Silico PCR tool were used to evaluate the specificity of the chosen primers. The table 2.3 shows the melting, product size, and primer order.

Table 2.3.: Primers for exon 2 of ARL6 gene.

GENE	EXON	FORWARD PRIMER (5′ ⟶3′)	REVERSE PRIMER(5′ ⟶3′)	PRODUCT SIZE (bp)	TM °C
BBS3/ARL6	2	GCTCCTTTCTGGTAATCAGCTT (Length 22 bp)	TGCAAATTATGGCTGAGGAT (Length 20 bp)	478	59

2.14. FASTA Sequence of Selected Exon 2 of ARL6:

TTAAATATGATAAATTACAGAGAGAAATGTTGTTATTTCCAAGGCATTGTTCTGTGTT
GCCACCTGTTATAAGCTGCAAATCTGCGTGTCAGTTAAGGCTCCTTTCTGGTAATCAG
CTTACACTGTGTTTACTAAGTGCAAAGCTACATTGACATAGTTTTCACACTATTATTA
TGTGTATTTAAATTATTACCTTTTTTTAATACACCTACCAATATTTTCCATAACTTAA
GGTGCCTTTGGGTAATATTTTATTTTTTCTTAATTGCAGCTGGTTTGTAAATATTTGA
ATCACATTATGGGATTGCTAGACAGACTTTCAGTCTTGCTTGGCCTGAAGAAGAAGGA
GGTTCATGTTTTGTGCCTTGGGCTAGATAATAGTGGCAAAACGACGATCATTAACAAA
CTTAAACCTTCAAATGTAAGTATCTTTGTTAGATGCTTTATGTATTTTCTGCTACTAA
AGAAAATTAATGTGCAGAATTATGTTATATGACGTTAAAACCGCATATAATCACATTA
AGATATTCTGTTAACCTTTCAGTACCTTTAAGTATCCTCAGCCATAATTTGCAGTATA
TTTTACACAGTAAATTTTAATTATTAAACTGGAATATTATTTTCAAATGTCTTTTCTT
TCAAAAGTTCTTCACAGATTTACTGAGTTTACTTTAAATGTCATTGCCTTCTTTTTAG
TTTCCTCCTTACAGGAGGAGCAAAACAGAGAAAGGTGAAACATTTTTATAGGTTCTAT
GGATAATTAGACAATTTTTGAAAGTATAGTATGCATATTAGTAATAGTAAGCAAGATG
ATTTTAAGCAACAAGTGTATGGACATATTGTAATAACTGAAAAATATATAAAGCTAGC
ACATTTAACATGTGATGTATAAGTTAAAGTTAAAGTCTGCTGCATATAAAGAAAGCCT
ATCTAATAGGGCTTTCCTAATTTCTGGCACCAATTCCTGAGCCTTTATGTTTGGACAA
CTCTCCTGCCTTATCAGAAAAACTGACCTCCAGGTCCAGAGAAAAGTGATTGGCTTTG

2.15. In-Silico PCR of Selected Primer for Exon 2:

>chr3:97767907+97768384 478bp

GCTCCTTTCTGGTAATCAGCTT

TGCAAATTATGGCTGAGGAT

GCTCCTTTCTGGTAATCAGCTTacactgtgtttactaagtgcaaagctacattgacatagttttcacactattattatgtg

tatttaaattattacctttttttaatacacctaccaatattttccataacttaaggtgcctttgggtaatattttatttttttcttaattgcagctggtttgt

aaatatttgaatcacattatgggattgctagacagactttcagtcttgcttggcctgaagaagaaggaggttcatgttttgtgccttgggcta

gataatagtggcaaaacgacgatcattaacaaacttaaaccttcaaatgtaagtatctttgttagatgctttatgtattttctgctactaaaga

aaattaatgtgcagaattatgttatatgacgttaaaaccgcatataatcacattaagatattctgttaacctttcagtacctttaagtATCC

TCAGCCATAATTTGCA

2.16. Primer Dilutions:

The ordered primers were diluted to achieve values of 10 picomole/ul from their initial concentrations of 100 picomole/ul.

2.17. Polymerase Chain Reaction:

The selected Exons 2 of the BBS3/ARL6 gene were amplified for six families using polymerase chain reaction in PCR tubes with a 200-ul capacity (Axygen, USA). For PCR amplification, the kit (Thermo-Scientific PCR Kit) was used. The table 2.4 below lists the chemicals and volume used in the PCR experiment.

Table 2.4: Chemicals used in PCR Mixture

S. No	Chemicals	Concentration	Volume (µl) for single PCR Reaction
1	DNTPs	2.5mM	2.5µl
2	MgCl2	25Mm	2µl
3	DNA	>1OOng/ul	2µl
4	Forward Primer	10 picomole/ul	0.5µl
5	Reverse Primer	10 picomole/ul	0.5µl
6	Taq. Buffer [(NH4)SO4]	10X	2.5µl
7	PCR Water		14.5 µl
8	Taq Polymerase	5U/ µl	0.5µl
	Total Volume		25 µl

The PCR tubes were briefly spun in the microfuge at 3000 rpm for one minute to mix the contents before putting them in the thermos cycler (Bio-Rad T100) for the PCR reaction. The temperature settings for the PCR process are listed in the table 2.5 below.

Table. 2.5.: Conditions for PCR Cycles

Step	Temperature	Time	Cycle
Initial Denaturation	96 °C	5 min	1X
Denaturation	95 °C	45 sec	40X
Annealing	65-55 °C	45 sec	
Extension	72 °C	90 sec	
Final Extension	72 °C	10 min	1X
Hold	25 °C	∞	

2.18. Confirmation of PCR product:

A 2% agarose gel was made by mixing 1g of agarose powder with 50ml of IX TBE buffer, and 2l of ethidium bromide was added during the gel preparation process in order to verify the PCR results. After that, a 2% agarose gel was permitted to run these DNA samples for 20 min at 120V. The gel was then examined utilizing the Gel Documentation System (Cleaver Scientific Limited) to verify the amplified portion of the BBS3/ARL6 Gene.

2.19. Purification of PCR Product:

PCR product was purified by using purification kit (Thermo-Scientific). The procedure applied for purification in given below.

- PCR product was transferred to 2.5ml Eppendorf tubes.
- Our PCR product was almost 20µl so we took 100µl of Binding buffer that was 5 times the volume of PCR product. These were mixed properly.
- The mixture was moved to a labelled spin column that was attached to a collection tubes that were also labeled.
- The mixture was centrifuged for two minutes in a Beckman Coulter Microfuge at 13000 rpm.
- Each sample received 350µl of washing buffer, which was then centrifuged again for one minute at 13000 rpm. The filtrate was discarded.

- The PCR products were completely purified by repeating steps 5.
- The filtrate was completely discarded.
- Each sample received 13µl of elution buffer. Before usage, the elusion buffer was maintained in an incubator at 70°C.
- In Eppendorf tubes with pre-attached labels, the spin column tubes were put.
- The samples were maintained for two minutes at room temperature.
- For one minute, tubes were centrifuged at 13000 rpm.
- To verify the purity of the PCR products, samples were tested on 2% Agarose Gel.

2.20. Sequencing:

Sanger sequencing method was used for the sequencing of the amplified PCR product. Each eppendorf tube included about 10 l of the purified PCR product, along with 30 l of the forward and reverse required primer (10 picomoles/l) of the chosen exons for sequencing. The tubes were carefully labeled, sealed, and shipped for commercial sequencing. Big Dye Terminator chemistry (used with an automated ABI PRISM 3730 Genetic analyzer) was used to carry out the sequencing procedure. The capillary electrophoresis method was used to separate the tagged DNA fragments, and a spectrum analyzer was used to confirm their detection. Each nucleotide (thymine, guanine, cytosine, and adenine) was marked with a particular dye for documentation purposes.

2.21. Mutation Analysis:

The sequenced information was compared to a reference sequence from the Ensemble genome browser (https://asia.ensembl.org/Homo sapiens/Info/Index). In Bio-edit (v.7.2.0), the sequences were entered, and they were aligned with the reference sequence. The dispute was tested using a mutation tester. The chromatogram was examined using Chromas 2.66.

CHAPTER.3

RESULTS

3. RESULTS

3.1. Clinical characteristics:

Blood samples from 25 families belonging from various regions of Khyber Pakhtunkhwa were collected at Hayatabad Medical Complex and Khyber Teaching Hospital Peshawar. At least two people in each family had been given a Retinitis Pigmentosa diagnosis, and all families had a history of the condition. Initially families were enrolled on the basis of RP symptoms that were diagnosed by Ophthalmologist. Also fundus photographs of 15 families were taken. After detailed interview Bardet-Biedl syndrome families were separated. From each family, a proband was chosen for exon 2 of the ARL6 gene mutation analysis. Every family that possesses a certain combination of the primary and secondary BBS characteristics specified in tables 3.1 and 3.2.

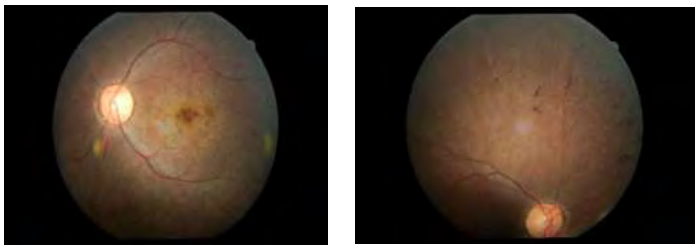

Figure 2.1: Representative of fundus photographs of BBS patients

Table 3.1: Primary Symptoms Observed in Proband of each selected BBS Family

BBS Features in Families Chosen For Genetic Analysis					
		Family ID/proband			
S.no	**Primary Features**	**RP-139/V-1**	**RP- 152/ IV-IV**	**RP-153/ III-XIX**	**RP-157/III-XIII**
1	Rod-Cone Dystrophy	YES	YES	YES	YES
2	Polydactyly	YES	YES	NO	YES
3	Obesity	YES	NO	YES	NO
4	Learning Problem	NO	YES	YES	YES
5	Hypogonadism	NO	NO	NO	NO
6	Renal Defects	NO	NO	NO	NO

Table 3.2: Secondary Symptoms Observed in BBS Families

BBS Features in Families Chosen For Genetic Analysis					
		Family ID/proband			
S.no	**Secondary Features**	**RP-139/V-1**	**RP- 152/ IV-IV**	**RP-153/ III-XIX**	**RP-157/III-XIII**
1	Liver Disease	NO	NO	NO	NO
2	Heart Problem	NO	NO	NO	NO
3	Hearing Issue	NO	YES	YES	YES
4	Dental Issues	NO	NO	NO	YES
5	Speech Problems	NO	NO	YES	NO
6	Developmental Delay	NO	NO	YES	NO
7	Diabetes Mellitus	NO	NO	NO	NO
8	Family Members with BBS	YES	YES	NO	YES

3.2. Family RP-139:

The family RP-139 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 20 members having three affected members. All the affected members were males (IV-2, IV-5 and IV- 7). The descriptive data was collected from all alive members. The ages of the effected alive members at the time of enrollment were 22, 25 and 33 years respectively. All the effected members of the family had progressive night blindness, visual impairment and bilateral RP. This family has characteristics of BBS syndrome. The proband had polydactyl condition along with RP. The pedigree shows the pattern of disease as autosomal recessive.

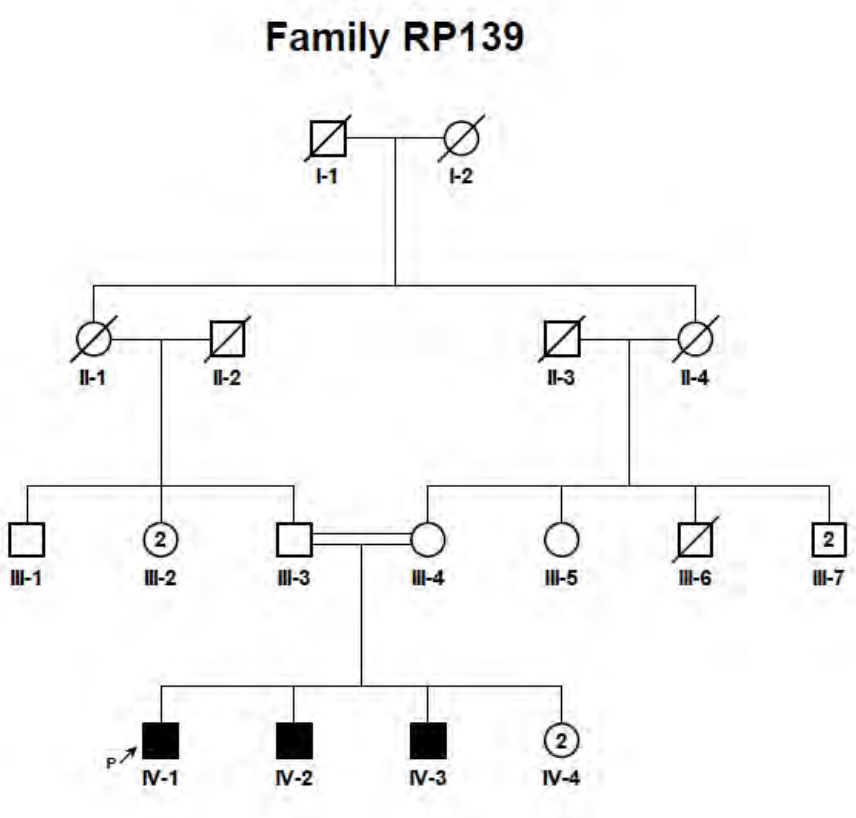

Figure 3.2: Pedigree of RP-139 Family

3.3. Family RP 152:

The RP family 152 belongs from Nowshera Khyber Pakhtunkhwa. This family consist IV generations having 31 members out of which 27 are alive and 4 are deceased. Two male members (IV-I and IV IV) and two female members (IV-II and IV- V) are diagnosed with RP) as shown in fig 3.1. The primary and secondary features of BBS possessed by the proband IV-IV) are listed in table 3.1 and 3.2. This family shows an autosomal recessive mode of inheritance but further genetic analysis is required to confirm the pattern of inheritance. Affected members (IV -1, IV-II, and IV-III) also possess BBS signs.

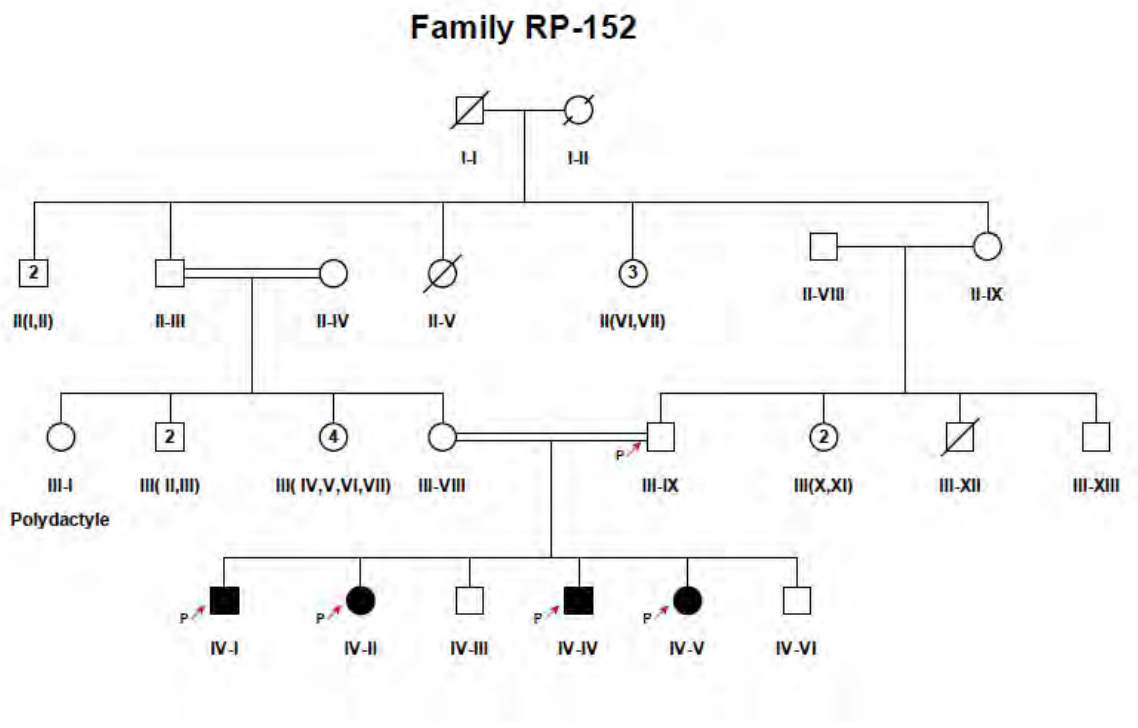

Figure 3.3: Pedigree of RP-152 Family

3.4. Family RP-153:

The family RP 154 consists of IV generations having 47 members out of which 42 are alive and 5 are deceased. This family consists of five affected members and belongs from Peshawar. This family consists of five affected members, four are males (III-I, III-II, III-XII, IV-1) and one female (III-XIX). All the affected members are diagnosed with RP and one member also possess the signs of BBS. After conducting a detailed interview with proband (III-XII), the sister of proband (III-XIX) was assigned BBS and its genetic analysis was performed because it was possessing BBS primary and secondary features as mentioned in table 3.1 and 3.2. At the time of enrollment the age of proband was 36 years while his son who was assigned BBS characteristics was 7 years old. This family depicts an X-linked dominant pattern of inheritance but it is not confirmed and further genetic analysis is required to confirm the pattern of inheritance.

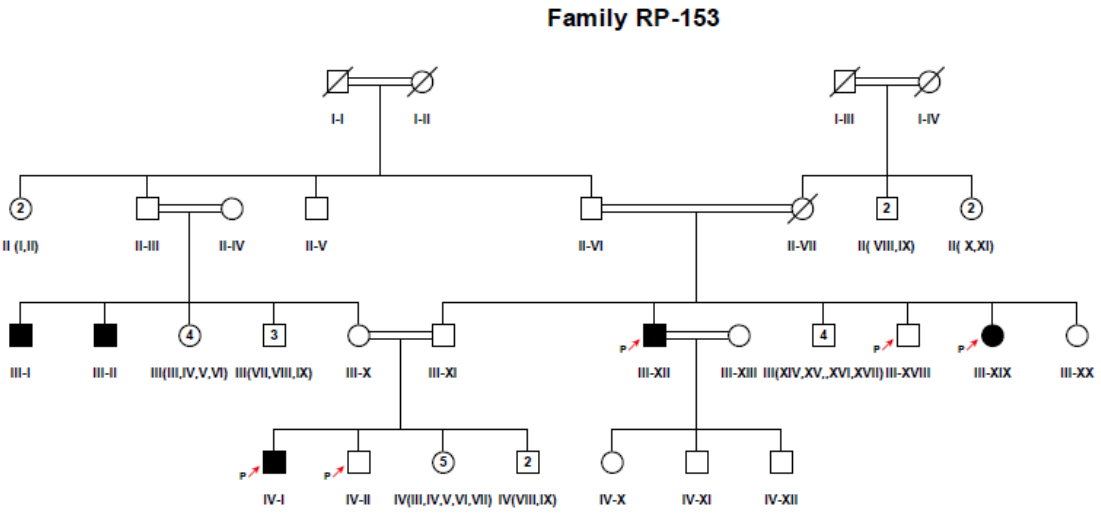

Figure 3.4: Pedigree of RP-153 Family

3.5. Family RP-157:

The RP family 157 belongs from Nowshera KPK. This family consist IV generations having 38 members out of which 30 individuals are alive and 8 are deceased. One male members (III-XIII-1) and one female member (III-XII) have complete symptoms of BBS that are shown in pedigree fig 3.3. Three members of the family have RP (III-XII, III-XIII, and IV-IV). This family shows an autosomal recessive pattern of inheritance. III-XIII proband of the family RP 157 was selected for genetic analysis. At the time of enrollment the age of proband III-XIII was about 24 years and the other affected member III-XII age was about 28 years old.

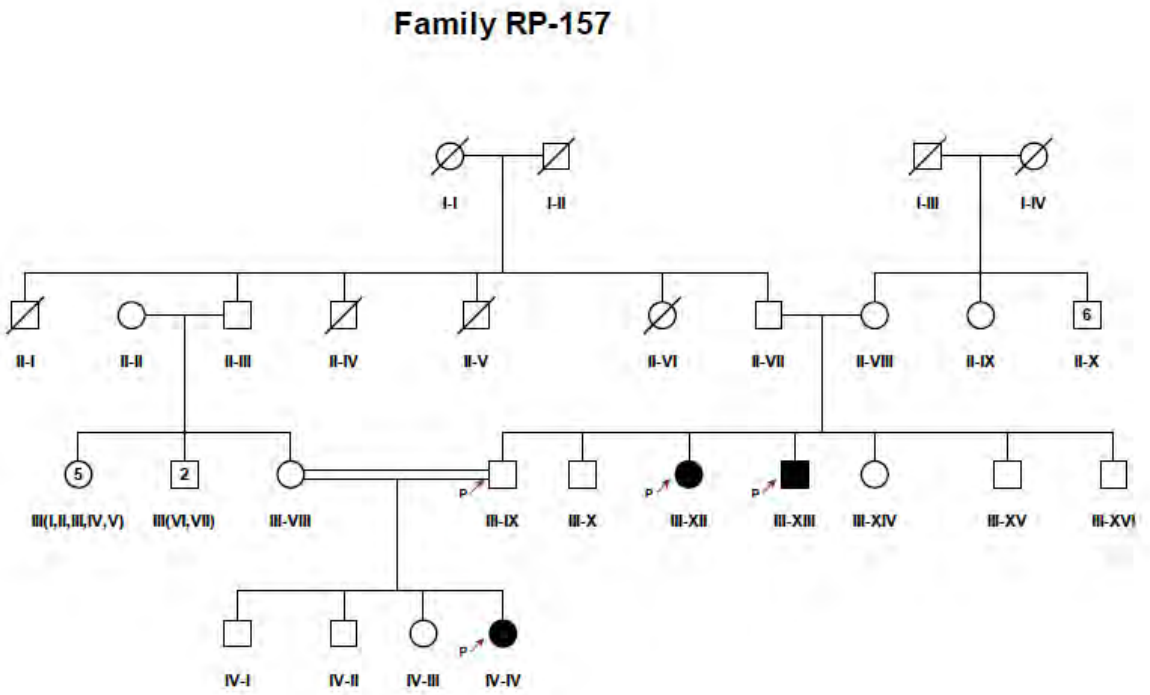

Figure 3.5: Pedigree of RP-157 Family

3.6. DNA Isolations:

The DNA was isolated from each blood sample using phenol chloroform method. The mean concentration of isolated DNA was up to **146.33 ng/µl** with purity value of **1.69** for each isolated sample.

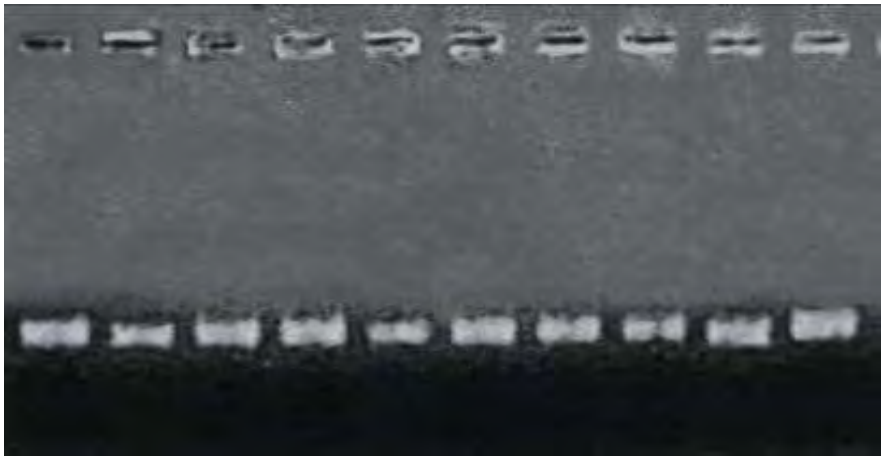

Figure 3.6: DNA Isolated from BBS Families

3.7. Primer Optimization:

For primer optimization gradient PCR was used. The annealing temperature, 55℃ were found for primer pair design for mutation analysis of mutation in exon 2 of ARL6 gene which is associated with BBS syndrome.

3.8. Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) was performed to amplify the selected exon 2 of *ARL6* gene of probands of collected BBS families. The product size of the primer used to amplify exon 2 of ARL6 gene is 478 bp. The confirmation of amplification was done by running 2% agarose gel electrophoresis and visualizing the gel in gel documentation system (Cleaver Scientific Limited) as shown in fig 3.8.

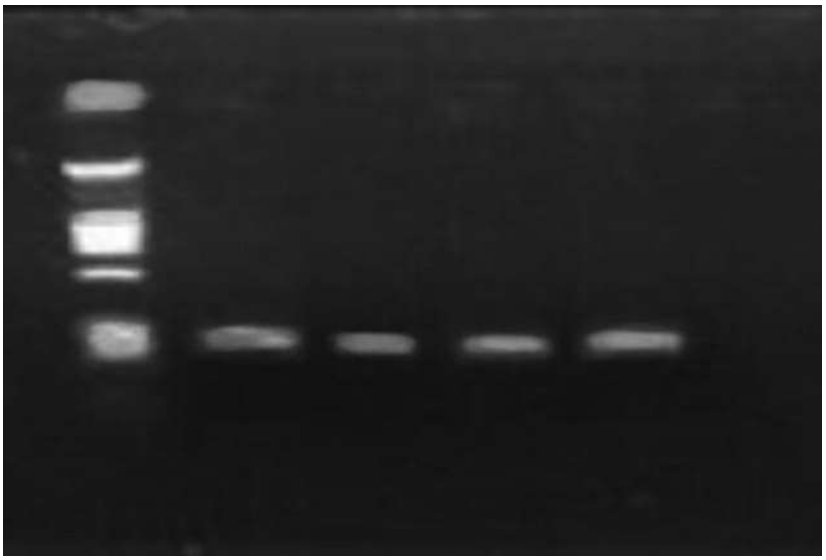

Figure 3.7: PCR Amplification results on 2% Agarose gel

3.9. PCR Product Purification:

For the purification of PCR product, a purification kit (Thermo-Scientific) was used. All the PCR product of probands was purified and 13µl purified PCR products were obtained. The confirmation of amplification was done by running 2% agarose gel electrophoresis and visualizing the gel in gel documentation system (Cleaver Scientific Limited) as shown in fig 3.8.

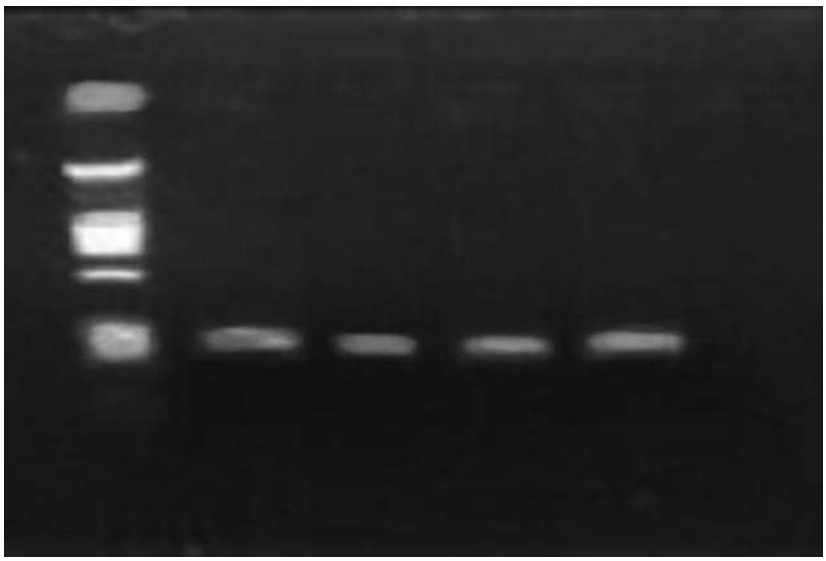

Figure 3.8: Confirmation of Purified PCR product

3.10. Sanger Sequencing:

All the purified samples were sent for Sanger's sequencing to Macrogen, South Korea. To accomplish the sequencing reaction Big Dye Terminator chemistry (an automated ABI PRISM 3730 Genetic analyzer) was applied. The labeled DNA fragments were separated using the capillary electrophoresis technique and were detected using a spectrum analyzer. For documentation purposes, each nucleotide (thymine, guanine, cytosine, and adenine) was labeled using a specific dye.

3.11. Genetic Analysis

Mutations in *ARL6* gene are known to cause Bardet-Biedl syndrome in different popullations globally.Exon 2 of *ARL6* gene (Ensemble Transcript I.D: ENST00000463745.6) was selected for genetic analysis of choosen families. *ARL6 having* Ensemble Transcript I.D: ENST00000335979.6 was used as standard for sequence alignment. All the analyzed sequences are listed below.

3.11.1. Exon 2 Analyzed Sequence of BBS selected families:

Sanger sequencing of exon 2 of ARL6 gene of selected proband of BBS families no mutation or change in the DNA sequence. The sequence of chromatogram is shown in fig 3.9.

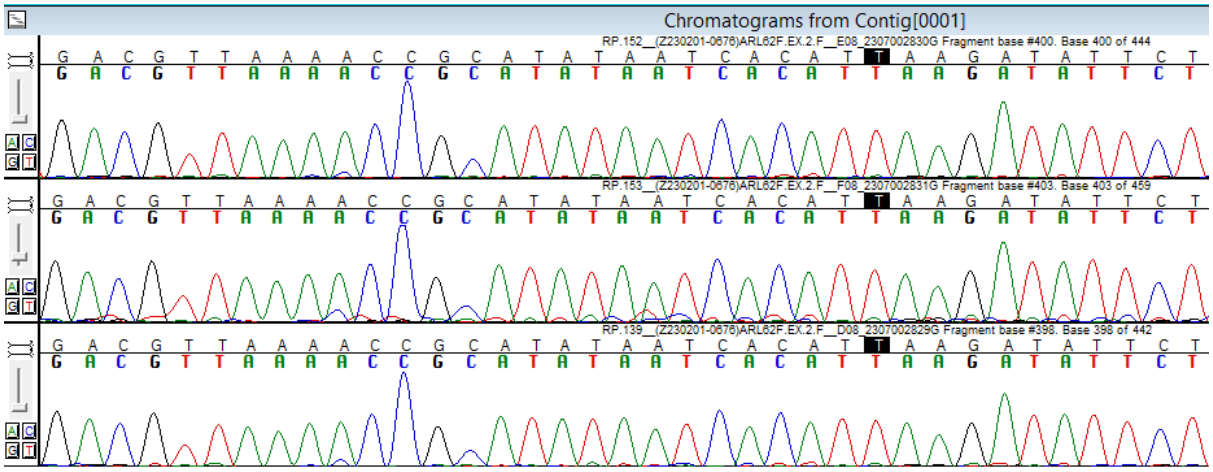

Figure 3.9. Sequencing Chromatogram: Showing no mutation in exon 2 of ARL6 gene of proband of RP-139, RP-152 and RP-153

CHAPTER.4
DISCUSSION

4. DISCUSSION

Ciliopathies are a class of diseases caused by primary cilium dysfunction, which can manifest as a variety of clinical conditions frequently involving retinal photoreceptors (Reiter and Leroux, 2017). The highly specialized organelle known as the cilium is present in eukaryotic cells and is essential for cell communication and movement (Goetz and Anderson, 2010). Cilia are classified into motile and non-motile (primary) subtypes (Wallmeier *et al.,* 2020). Non-syndromic retinitis pigmentosa, which can be inherited in autosomal dominant, autosomal recessive, and X-linked patterns, is a typical illustration of an isolated ciliopathy (Estrada-Cuzcano *et al*., 2012). Syndromic ciliopathies include Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS), Alström syndrome, Joubert syndrome, Usher syndrome, Senior-Løken syndrome and Cohen syndrome, all of which typically involve other organs in addition to the retina.

Bardet-Biedl syndrome (BBS) is one of the well-studied, pleiotropic syndromic ciliopathies due to (Forsythe *et al*., 2018). The classical symptoms of BBS include retinal degeneration with a mix of extraocular features comprised of renal disease, intellectual disability, truncal obesity, postaxial polydactyly and hypogonadism. According to the criteria outlined by Beales et al., a clinical diagnosis is made based on the presence of four main features or any three major traits along with two minor features. (Sheffield, 2016).

In populations that are inbred or consanguineous, BBS is more common. BBS prevalence in the general population is 0.7/100,000. In addition to a birth prevalence of 0.5 per 100,000 (Prevalence and Incidence of Rare Diseases: Bibliographic Data. Orphanet Report Series Number 1 January 2020). The prevalence of BBS varies, though: Bedouin populations make up 1 in 160,000 of the population in North America and Europe, 1 in 17,000 in Kuwait, and 1 in 3700 people in the Faroe Islands (Khan *et al*., 2016). BBS4 gene is reported as the most prevalent gene among Turks and Pakistanis (Fattahi et al., 2014). In Saudi Arabia, BBS1, BBS3, and BBS4 mutations are frequently documented (Abu Safieh *et al*., 2010). Although BBS1, BBS2, and BBS8 pathogenic mutations are frequently found in Tunisia (Smaoui *et al*., 2006). BBS4, BBS5, and TTC8 variations are prevalent in people from the Middle East and North Africa (Billingsley *et al*., 2011).

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Twenty-six genes have currently been linked to the condition attributable to advancements in genetics, a rising number over time (Meyer *et al.,* 2022). Genes are expressed in all tissues, but there are differences in expression levels. Rod photoreceptor cells (BBS1, ARL6, BBS4, BBS5, BBS7, TTC8, BBS9, BBS12, CEP290, NPHP1, and SCAPER) and ciliated cells (BBS1, BBS2, ARL6, BBS4, WDPCP, LZTL1, IFT27, IFT74, and NPHP1) have all been reported as having a cell type enhanced expression. Spermatocytes (ARL6, LZTFL1, BBIP1, IFT74, NPHP1), early spermatids (BBS5, BBS12, BBIP1, NPHP1), late spermatids (BBIP1), alveolar cells type 1 (WDPCP), alveolar cells type 2 (WDPCP), and club cells (WDPCP) are all examples of cone photoreceptor cells (BBS1, ARL6, BBS4, BBS5 TTC8, BBS9, SCAPER) (Proteinatlas the Human Protein Atlas. Available online: https://www.proteinatlas.org (accessed on 19 December 2019).

RAB, ADP-ribosylation factors (ARFs) and ARF-like (ARL) proteins belong to the Ras superfamily of small GTP-binding proteins and are essential for various membrane-associated intracellular trafficking processes (Pasqualato *et al*., 2002). Among Saudi BBS patients, there is an unusual predominance of ARL6/ARL6 gene mutations, all of which are novel, in striking contrast to the BBS reports from Caucasians in which ARL6 gene mutations are extremely rare (Abu Safieh *et al*., 2010). The identification of mutations only in the large previously linked BBS3 Bedouin family is consistent with previous reports of the rarity of BBS3 as a cause of this syndrome (Bruford *et al*., 1997). The reported tissue expression pattern of ARL6 (Jacobs et al. 1999) and analysis of ESTs from dbEST indicate that ARL6 is widely expressed in human tissues including brain, eye, heart, and kidney, further supporting this gene as a cause of BBS ((Li *et al*., 2004).

In present study three consanguineous families were enrolled, having origin of KPK. Total 25 families were collected who had RP symptoms and 6 out of 25 shows BBS symptoms. It accounts only 24%. These six families in total were explored in depth for this aim based on clinical presentation in accordance with the BBS phenotype. Each family has multiple effected individuals.

The most common reported major feature of BBS is retinal cone dystrophy (94%), which was also present in all selected BBS families (100%). The other major feature which was most common in BBS selected families was polydactyl (66%), while it is 79% according to Forsyth *et al.,* 2023. While obesity accounts for 89%, and only 33% obesity as major feature was present in selected BBS families. In addition, other feature like cognitive impairment accounts for 66% and in our selected BBS families it is also 66%. Other major features such as hypogonadism and renal defects was not observed in any family, merely because either male

or female have had biological children. The minor features observed mostly on BBS selected families were neurological abnormalities (66%) and dental abnormalities (33%)., while the reported data accounts for 81% neurological abnormalities and 50% oral and dental abnormalities (Forsyth *et al*., 2023).

According to (Beales et al., 1999) more than 90% of BBS patients have retinal cone dystrophy. Obesity accounts for 72–92% (Moore et al., 2005). Polydactyl 63–81% (Schaefer *et al*., 2014). The incidence of hypogonadism in BBS populations is about 59–98% (M'hamdi *et al*., 2014). Mental retardation accounts for about 50–61% in BBS patients (Forsthye *et al*., 2013). Renal abnormalities accounts for 20–53% of BBS patients (Raychowdhury *et al.,* 2009). Daibetes is about 6–48% in BBS patients (M'hamdi *et al*., 2014). There are extremely variable form of cardiac aberrations detected in BBS patients (Elbedour *et al*., 1994). Hearing present in about 11–12% of BBS patients (Ross et al., 2005). Symptoms of hypertension account for about 30% and 60%, respectively, in BBS patients (Imhoff *et al*., 2010). Speech deficits is about 54–81% in BBS patients (M'hamdi *et al*., 2014).

All the families included in the study shows Autosomal recessive mode of inheritance. Further genetic study is required for the confirmation of mode of inheritance of families. Although some publications have proposed an oligogenic inheritance, the BBS has an autosomal recessive inheritance (Manara et al., 2019). According to (Mykytyn *et al*., 2003), BBS1 has an autosomal recessive inheritance pattern and is infrequently, if ever, a component of complex inheritance. There are notable exceptions to the rule that inheritance is autosomal recessive, such as BBS, which may be an oligogenic condition (Katsanis *et al*., 2001). According to (Scheidecker *et al*., 2014), BBS has a high degree of locus and allele heterogeneity and segregates in an autosomal recessive manner.

The current study's objectives were to describe the demographic and clinical features of BBS patients in our local population and to conduct genetic analysis of the ARL6 gene to find sequence variants. The ages of enrolled effected individuals were from 7 to 35 years. In this study hotspot exon 2 was selected based on the previous studies in Pakistan. ARL6 and BBS10 are the most frequently mutated BBS genes in Pakistan, and these are mutational hotspots in Pakistan, according to reports on BBS gene mutations from Chen, Smaoui *et al*. (2011), Khan, Ullah *et al.* (2013), Maria, Lamers *et al*. (2016), Agha, Iqbal *et al*. (2013), Ullh, Umair *et al*. (2017), and Khan, Ullah *et al*, (2013). Only exons 2 of the ARL6 gene were chosen for genetic investigation in the current study based on funding availability.

Out of 6, only 4 families having BBS symptoms were selected because unavailability of funds and Sanger sequencing, of exon 2 of ARL6 gene was done. No variants were detected in exon 2 of ARL6 gene in those four families. While To date, 16 mutations have been reported in the gene BBS3/ARL6 causing BBS syndrome including splice site, missense, nonsense and deletion mutations (Khan *et al*., 2016). Compared to reports from other populations (0.4%), the ARL6 gene in the Indian population was responsible for 18% of the mutations (Sathya et al., 2015).

The most widely used approach nowadays is next-generation sequencing (NGS), which can be used for both research and diagnostic reasons. In order to determine the BBS molecular determinants in selected BBS families RP (139, 152 and 153), comprehensive sequencing of the ARL6 gene should be carried out in the future utilizing NGS technology.

All of the enrolled and highly inbred families received genetic counseling in an effort to reduce the prevalence of disease in future generations by discouraging cousin marriages in RP and BBS affected households. The results of this study call for more public education on the prevalence of inherited recessive disorders in Pakistani society in order to reduce disease burden as well as mortality and morbidity associated with such diseases in the future. Our community's experience with recessive genetic disorders may help researchers learn more about the disease's pathophysiology and develop novel therapeutic approaches.

4.1 Conclusion and Future Perspectives

In present study no mutations were observed in exon 2 of ARL6 gene. According to previous studies, 3% mutations are reported. In current study, the incidence of BBS families is 24%. In previous studies, very high incidence of BBS has been reported within two forms of populations, those having high rate of consanguinity and geographically isolated ones. To date, there is no specific therapy and supportive treatment is the milestone of patients' care. A multidisciplinary approach is mandatory in BBS, considering the presence of multiple organ dysfunction, and personalized follow-up is required. Moreover, genetic counseling was given to all the affected families to avoid transmission of autosomal recessive traits by consanguineous marriages that could lead to the incidence of syndromes like BBS. However, the study needs more research innovations and funding, where whole genome sequencing will bring more insight, as the disease is highly heterogeneous.

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Thesis

ORIGINALITY REPORT

16%	12%	11%	8%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	3%
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4	Bharatendu Chandra, Moon Ley Tung, Ying Hsu, Todd Scheetz, Val C. Sheffield. "Retinal ciliopathies through the lens of Bardet-Biedl Syndrome: Past, present and future", Progress in Retinal and Eye Research, 2021 Publication	1%
5	Khan, Sher Alam, Noor Muhammad, Muzammil Ahmad Khan, Anwar Kamal, Zia Ur Rehman, and Saadullah Khan. "Genetics of Human Bardet-Biedl Syndrome, an Updates", Clinical Genetics, 2016. Publication	1%
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ETHICAL APPROVAL CERTIFICATE

The Ethical Review Board of the Hayatabad Medical Complex has reviewed the under mentioned Synopsis/Article in accordance with the declaration of Helsinki (2013) and found it to meet the requirements and be approved.

Approval No:	**1018**
Synopsis / Research Article	**Study of Molecular Basis of Retinitis Pigmentosa in Pakistani Population.**
Investigators / Authors	**Sumbal Wazir**
Approval Date	**25/10/2022**
IREB Decision	**Approved**

Standard conditions of Approval Apply.
The Principal Researcher is required to notify the Secretary of the Ethical Committee about:

- Any significant change to the project and the reason for that change, including an indication of Ethical implication (if any).
- Serious adverse effects on participants and actions are taken to address those effects.
- Any other unforeseen events or unexpected developments that merit notification.
- The inability of the Principal Researcher to continue in that role, or any other change in research personnel involved in the project
- Provide the progress report / Final report / discontinuation report.

Chairman IREB / Secretary

Professor Dr. Wajid Ali Akhunzada
Chairman
Institutional Research & Ethical Board
Hayatabad Medical Complex Peshawar

Telephone No.
90643074

QUAID–I–AZAM UNIVERSITY
Faculty of Biological Sciences
ISLAMABAD, PAKISTAN

Ref. No. ZOO-2022/ Dated: 01.09.2022

TO WHOM IT MAY CONCERN

Subject: **Permission for enrollment of patients (data & blood sample collection) for Research purpose**

Postgraduate research projects of student of molecular biology laboratory at Department of Zoology, Quaid-i-Azam University Islamabad involve molecular genetic analysis of inherited eye disorders in local population. To achieve study objectives, she has to enroll clinically diagnosed patients affected with retinitis pigmentosa and xeroderma pigmentosum. The purpose of this letter is to request your kind permission and cooperation for patient's family and clinical data as well as blood samples collection from your hospital. Written consent will be taken from affected patients/their guardians as applicable. She has to enroll at least 30 patients with positive family history of disease in the course of six months. Details of student are as follow:

Student's Name & Registration No.

Sumbal Wazir (Reg. No: MP-F21-14384)

Thanking you in anticipation.

Sincerely,

Dr. Sabika Firasat
Research Supervisor

FACULTY OF BIOLOGICAL SCIENCES, DEPARTMENT OF ZOOLOGY

QUAID-I-AZAM UNIVERSITY, ISLAMABAD

LAB OF MOLECULAR BIOLOGY

Questionnaire and Consent Form

There are a number of eye diseases prevalent in our society on which insufficient information is available. Prevalence of different eye diseases is increasing day by day but their underlying causes are still less explored especially in our population.

We researchers of Quaid-i-Azam University, Islamabad with your kind information will obtain data on eye diseases segregating in your family. We will also need patient's clinical and family history, medical reports as well as 5 to 6ml of blood samples for molecular analysis. The researcher teem will keep your personal and medical information secret and will inform you regarding molecular analysis result upon study completion.

Researchers on molecular genetics characterization of prevalent eye diseases in local population will translate into improvement in the management and quality of care provided to affected individuals.

I consent to send DNA sample for processing at a collaborating foreign institute/s. The processing will include analysis of genetic makeup by Sanger's sequencing or next generation sequencing (NGS), which are unavailable at Quaid-i-Azam University.

I hereby certify that after reading above mentioned information, I with my family are willing to be a part of this research and will provide honest answers to whatever we are asked in the process. I, the undersigned, give my consent for genetic analysis using DNA from blood samples taken by researcher teem and publication of case history and research findings in a scientific journal/conference under the legislation of Pakistan, which at present does not include national data protection legislation.

Name …… D/O/S/O…….

CNIC …… Disease…….....

Phone number ……..……. Signature……...

Researcher's Signature ……...…. City/District/Province ……..…..

Address ……...……...……...……...…

FACULTY OF BIOLOGICAL SCIENCES, DEPARTMENT OF ZOOLOGY

QUAID-I-AZAM UNIVERSITY, ISLAMABAD

LAB OF MOLECULAR BIOLOGY

PROFORMA FOR RETINAL DYSTROPHY PATIENTS

Personal Information

Hospital ID	----------	**Date**	----------
Individual's Name	----------	**Father Name**	----------
Age	----------	**CNIC**	----------
Gender	----------	**Disease status**	**Affected / Normal**
Family	**RP/RB/Others**	**Family ID**	----------
Diagnosed Disease	----------	**Hospital**	----------
Type of Sample	----------	**Sample ID**	----------
Ethnicity	----------	**Profession**	----------
Contact Details	----------	**Address**	----------

Family History

Family History	**Positive / Negative**	**Consanguineous Parents**	----------
Relation of Parents	----------	**No of Affected Individuals**	----------

Anthropometric Data and general Information

Weight (Kg)	----------	**Height (cm)**	----------
Head circumference (cm)	----------	**Initially Complaint**	----------
Age of Onset	----------	**Current Vision**	----------
Any Diagnostic Test	----------	**Any Medical Surgical Treatment**	----------
Photophobia	----------	**Nystagmus**	----------
Color Perception	----------	**Light Perception**	----------
Cataract	----------	**Micropthalmia**	----------
Anapthalmia	----------	**Corneal Opacity**	----------
Blindness	----------	**Onset of Blindness**	----------
Infectious Eye Disease in Past	----------	**Currently Infectious Eye**	----------
Hearing	----------	**Speaking**	----------
Facial Dysmorphism	----------	**Teeth Structure and Count**	----------
Bone Deformities	----------	**Infertility/Fertility/Unmarried**	----------
Number and Structure of Hand's Fingers	----------	**Number and Structure of Foot's Fingers**	----------
Any other disease	----------	**Other Assessments (Speech, intellectual, behavior etc.)**	----------
Visual Acuity	----------	**Visual Field**	----------
Fundoscopy	----------	**ERG**	----------
MRI/CT Scan	----------	**Any Other Findings**	----------

Clinical Information

1. **V/A$_{SC}$ (Vision Assessment without Glasses):**

2. **V/A$_{CC}$ (Vision Assessment with Glasses):**

3. **Refractive Error:**

   i. Hyperopia **ii. Myopia** **iii. Astigmatism**

4. **Signs and Symptoms:** _____

5. **Age of Onset:** _____

6. **Associated Systemic Features:** _____

7. **Eye Examination:**

   a. Nystagmus: Yes / No

   b. Keratoconus: Yes / No

   c. Squint: Yes / No

8. **Intraocular Pressure (I.O.P):** _____

9. **Anterior Segment Findings:** _____

_____

10. **Retinal Findings:**

    a. Disc: _____

    b. Macula: _____

    c. Pigmentary Changes: _____

Collected samples

Sr #	Name	Patients/control	Sample ID (EDTA Number)	RP Family ID

Fundus photographs

Pedigree