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Sanger Sequencing of *BPY2* gene of AZFc Region among Selected Bangladeshi Azoospermic Male Infertile Patients

Dr. Masuma Rashid Raka[1*], Prof. Laila Aujuman Banu[2], Dr. Md. Rezaul Karim[3]. Dr. Md. Ashif Iqbal[4]

1. Assistant Professor & Head, Department of Anatomy, Mandy Dental College, Dhaka, Bangladesh.
2. Chairman, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh
3. Assistant Professor, Department of Anesthesiology, Dhaka Dental College, Dhaka, Bangladesh.
4. Research Assistant, Department of Urology, National Institute of Kidney Diseases & Urology (NIKDU), Dhaka, Bangladesh.

Abstract:
Background: *Male infertility is a complex disorder with both genetic and environmental contributors, and Y chromosome microdeletions, particularly in the AZFc region, are among the most well-established genetic causes. The BPY2 gene, located within the AZFc region, plays a crucial role in spermatogenesis, and its deletions have been associated with azoospermia. This study aimed to investigate BPY2 gene variations (Exon4 to Exon8) among Bangladeshi azoospermic male infertile patients using Sanger sequencing.* ***Methods:*** *A total of 64 azoospermic male patients were recruited, and genomic DNA was extracted from peripheral blood samples. Sanger sequencing was performed for Exon4 and Exon5 of BPY2 gene, and sequence alignment was conducted using BLAST against the NCBI reference sequence to detect mutations or deletions.* ***Results:*** *The sequence analysis showed 100% similarity with the NCBI reference sequence, with no detectable mutation or deletion in Exon4 and Exon5 among the study population. These findings suggest that BPY2 exon variations are not a major contributing factor to azoospermia in Bangladeshi men.* ***Conclusion:*** *This study provides important insights into the genetic profile of Bangladeshi azoospermic men, indicating that BPY2 gene exon mutations are not a primary cause of azoospermia. However, as spermatogenesis is a multifactorial process, further research involving whole-genome sequencing, regulatory region analysis, and haplogroup characterization is needed to explore other genetic influences. Expanding genetic screening approaches could enhance the diagnosis and management of male infertility in diverse populations.*

Key words: *BPY2, AZFc, azoospermia, male infertility, Sanger sequencing, Bangladeshi*

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I. Introduction:

The human Y chromosome harbors a number of genes that plays a crucial role in sex determination and male fertility. Many of these genes are directly or indirectly involved in spermatogenesis, making the Y chromosome a key genetic determinant of male reproductive health [1]. Approximately 10 mega bases (Mb) of the Y chromosome consist of complex amplicon regions, composed of highly repetitive DNA sequences that share 99.8% sequence identity between copies [2, 3]. Most genes exhibiting testis-specific or predominant expression, except SRY, are found within these amplicons and exist in multi-copy gene families [2]. However, the repetitive structure of the Y chromosome renders it highly susceptible to nonallelic homologous recombination (NAHR), leading to duplications and deletions [4].

It is very difficult to determine the genetic component of male infertility [5]. Tiepolo and Zuffardi (1976) [6] first reported the involvement of Y-chromosome in male infertility. There is a region on the long arm of Y chromosome which is required for spermatogenesis and named as the azoospermia factor (AZF) region. Genes located in the AZF regions are potential candidates for genes involved in spermatogenesis [7]. Deletions in the azoospermia factor (AZF) region on the Y chromosome have been considered one of the major genetic causes of male infertility [8]. AZF has three subregions: AZFa, AZFb and AZFc [9]. The AZF region of the Y chromosome possesses a high mutation rate [10]. The most frequently deleted region is AZFc [11]. AZFc involves a cluster of genes which are required for spermatogenesis [12].

Basic protein human on Y chromosome 2 (*BPY2*) gene (GenBank accession no.: AC006366) is one of the gene that is located in the long arm of Y chromosome (Yq11.223) starts from base pair 22,984,263 to 23,005,465 (National Center for Biotechnology Information [NCBI] 2021) within the AZFc region in men. *BPY2* gene is

composed of nine (9) exons spanning 21 kb with the first ATG codon within exon4 and a termination codon within exon8 [13] and it encodes a protein of 106 amino acids (OMIM 400013). The encoded protein interacts with ubiquitin protein ligase E3A, which are the enzymes that degrade other proteins within cells. These enzymes attach a small protein called ubiquitin to other proteins that should be degraded [14]. It is involved in male germ cell development and any alteration here may lead to spermatogenic failure and ultimately may lead to male infertility [15].

Interestingly, men with AZFc deletions, have a 75% chance of having sperm identified on testicular biopsy [16]. Intra cytoplasmic sperm injection (ICSI) is a good option for this couple. But sons conceived after ICSI have a high chance of having impaired spermatogenesis. So appropriate genetic counselling is necessary to explain and to inform them about the possible alternatives or additional treatments, such as preimplantation genetic diagnosis to select female embryos [17].

The sequence analysis will help to counsel the patients appropriately about the treatment options and risk to offspring. Now-a-days current urologic intervention can provide a pathway to fertility for some patients with chromosomal abnormalities. For example, clustered regularly interspaced short palindromic repeats (CRISPR) technology is a gene editing tool. CRISPR allows removal of genes in chromosomes or complete removal of an autosome or sex chromosome [18]. It may allow individuals with virtually any chromosomal abnormalities to have the chromosomal abnormality repaired, hopefully improving chances of fertility [19].

Sanger sequencing is a gold-standard technique for precise DNA analysis, widely used to detect mutations, deletions, and structural variations in targeted genes. Its high accuracy makes it particularly effective for investigating genetic disorders, including those linked to male infertility. In this context, Sanger sequencing facilitates detailed analysis of AZFc genes, including *BPY2*, to identify potential mutations or sequence variations contributing to azoospermia and impaired spermatogenesis [20]. Compared to high-throughput sequencing, it offers a high-fidelity, targeted approach, making it essential for studying Y-linked fertility genes [21].

This study employs Sanger sequencing to characterize the *BPY2* gene within the AZFc region among Bangladeshi azoospermic male patients. By examining structural variations, mutations, and sequence integrity, the study aim to elucidate *BPY2*'s role in azoospermia and its impact on male fertility. The findings may contribute to improved genetic screening and diagnostic strategies for azoospermic patients.

II. Methodology:

Study Design: This cross-sectional genetic study aimed to investigate Sanger sequencing of the *BPY2* gene in the AZFc region among Bangladeshi azoospermic male infertile patients. The primary objective was to identify potential genetic variations in Exon4 to Exon8 of *BPY2* gene that may contribute to azoospermia.

Study Population and Sample Size: This study included 64 azoospermic male infertile patients, recruited from the Outpatient Department of Reproductive Endocrinology and Infertility, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. Patients were eligible if they were 21–50 years old, had a confirmed diagnosis of azoospermia based on semen analysis, had no history of obstructive azoospermia, and provided informed consent. Patients were excluded if they had chronic systemic diseases (e.g., diabetes, hypertension), a history of gonadal infections, chemotherapy or had previously received hormonal treatment for infertility.

Laboratory Procedures

1. Sample Collection and DNA Extraction

Peripheral blood samples (2 mL) were collected under sterile conditions in EDTA tubes. Genomic DNA was extracted using the ReliaPrep™ DNA Extraction Kit (Promega, USA) following the manufacturer's protocol. DNA quality and concentration were assessed using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). Samples with an A260/A280 purity ratio of 1.7–1.9 and a concentration ≥20 ng/µL were selected for further analysis.

2. PCR Amplification of *BPY2* Gene (Exon4 to Exon8)

Primers targeting Exon4 to Exon8 of *BPY2* gene were designed using Primer3 Plus and validated with OligoAnalyzer Tool (Integrated DNA Technologies, IDT).

The sequences of primers are-

Exon4:

Forward primer 5'- GGCCAATTGCCTAAGTGAAG -3'

Reverse primer 5'- CCGAGAACCTGTTAGGACACA-3'

Exon5:
Forward primer 5'- TCTTTGGCCCATTTTCTAGG-3'
Reverse primer 5'- AAACCTGGTGGTGAGAGGAA-3'

Exon6 & exon7:
Forward primer 5'- CCGCTGATTGAAAAACCTACA -3'
Reverse primer 5'- GTTGGCAGAACCCAAAAAGA-3'

Exon8:
Forward primer 5'- TTGTTTCCCATGTGTGCATT-3'
Reverse primer 5'- TCAGGACCTGGGCTAACTTTT-3'

PCR was performed using Hot Start® PCR Master Mix (Promega, USA), with reaction conditions optimized for each exon. The PCR products were verified through 1% agarose gel electrophoresis stained with ethidium bromide. Amplicons were visualized under a UV transilluminator, with expected product sizes of 457 bp for Exon4 and 300 bp for Exon5. Exon6, Exon7, and Exon8 were not amplified in this study.

3. Sanger Sequencing: Successfully amplified Exon4 and Exon5 products were purified and sequenced using the ABI-3500 Genetic Analyzer (Thermo Fisher Scientific, USA) with the BigDye® Terminator v3.1 Cycle Sequencing Kit.
Sequence and Mutation Analysis Workflow:

1. Sequence Data Processing: Raw sequencing data were analyzed using Chromas software.
2. Alignment with Reference Sequence: The obtained sequences were aligned with the NCBI reference sequence of *BPY2* gene (GenBank accession no.: AC006366) using BLAST analysis.

Statistical Analysis: Descriptive statistical analysis was conducted using SPSS (IBM SPSS Statistics, version 25, 2017). Data were presented as mean ± standard deviation (SD). BLAST results were used to compare sequence variations, and findings were analyzed for potential associations with azoospermia.

Ethical Considerations: The study was approved by the Institutional Review Board (IRB) of BSMMU, and written informed consent was obtained from all participants. Confidentiality was maintained by assigning unique patient ID numbers to anonymize data.

III. Result:

Table 1: Sociodemographic and Clinical Characteristics of Azoospermic Male Infertile Patients in the *BPY2* Gene Sequencing Study

Characteristic	Number of Patients (n = 64)	Percentage (%)
Age at Diagnosis		
21-30 years	20	31.3
31-40 years	36	56.3
41-50 years	8	12.5
BMI		
Normal (18.5–25)	34	53.1
Overweight (25-30)	26	40.6
Obese (>30)	4	6.25
Education		
Secondary School	38	59.38
Higher Education	36	40.63
Occupation		
Service Holder	36	40.63
Businessman	24	37.5
Worker (e.g., factory)	24	21.88
Exposure History		
Positive	8	12.5
Negative	56	87.5
Family History of Infertility		
Positive	4	6.25
Negative	60	93.75

Among the 64 azoospermic male patients, the majority (56.3%) were diagnosed between 31–40 years, with most having a normal BMI (53.1%) or being overweight (40.6%). Educationally, 59.38% had secondary schooling, and 40.63% had higher education. Service holders (40.63%) and businessmen (37.5%) formed the largest occupational groups. Only 6.25% had a family history of infertility, suggesting that genetic factors like *BPY2* gene variations may be less frequent but still relevant. 87.5% had no known exposure history, reducing the likelihood of acquired infertility causes. These characteristics help in assessing genetic contributions to azoospermia in this study. (Table 1)

The mean concentration of extracted DNA was 49.43 ± 23.93 ng/µL, with a mean purity ratio (A260/A280) of 1.80 ± 0.093. A total of 87.50% of DNA samples had an acceptable purity range (A260/A280 = 1.7–1.9), indicating high-quality DNA suitable for PCR amplification and sequencing (Figure 1).

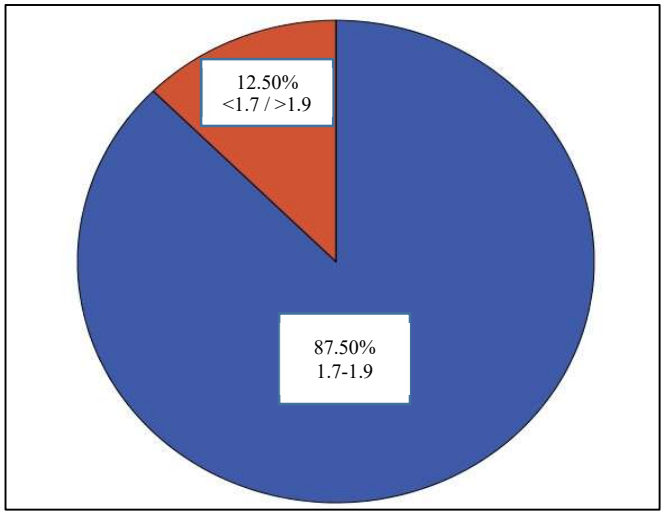

Figure 1: Distribution of Extracted DNA Samples Based on A260/A280 Purity Ratio

Following PCR amplification, the integrity and quality of the amplicons were assessed using 1% agarose gel electrophoresis. The analysis confirmed successful amplification of Exon4 (457 bp) and Exon5 (300 bp), as indicated by distinct bands on the gel (Figure 2). However, Exon6, Exon7, and Exon8 were not amplified, as no corresponding bands were detected. A 100 bp DNA ladder was used as a reference, and the negative control (lane 9) showed no amplification, confirming the absence of contamination in the experiment.

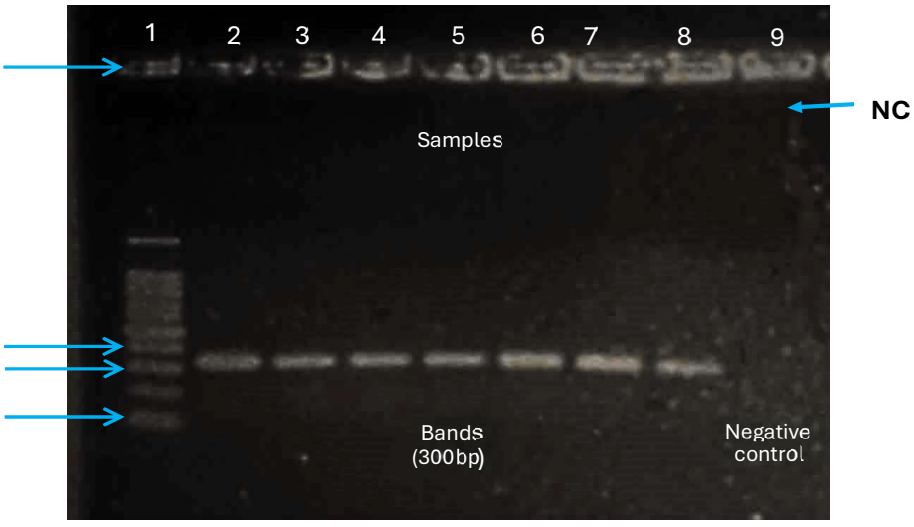

Figure 2: Gel electrophoresis of the PCR products on 1% agarose gel

Sanger sequencing was performed on Exon4 and Exon5 of the *BPY2* gene, and the resulting sequences were aligned with the NCBI reference sequence using BLAST analysis. The sequencing results confirmed 100% sequence similarity, indicating no mutations or genetic variations in
Exon4 and Exon5 among the 64 sequenced samples (Figure 4). The Sanger sequencing chromatogram further validated sequence integrity, confirming accurate amplification and sequencing (Figure 3).

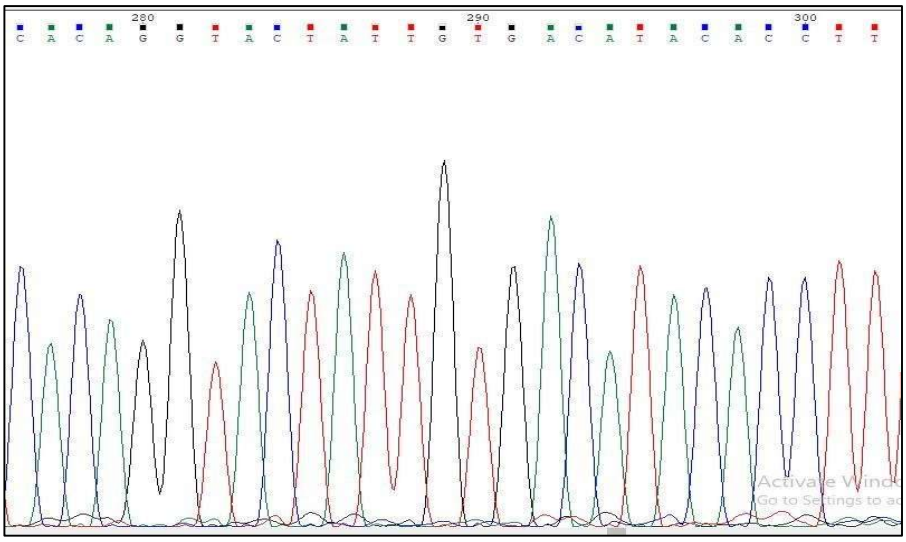

Figure 3: Chromatogram Showing *BPY2* Gene Sequence Analysis

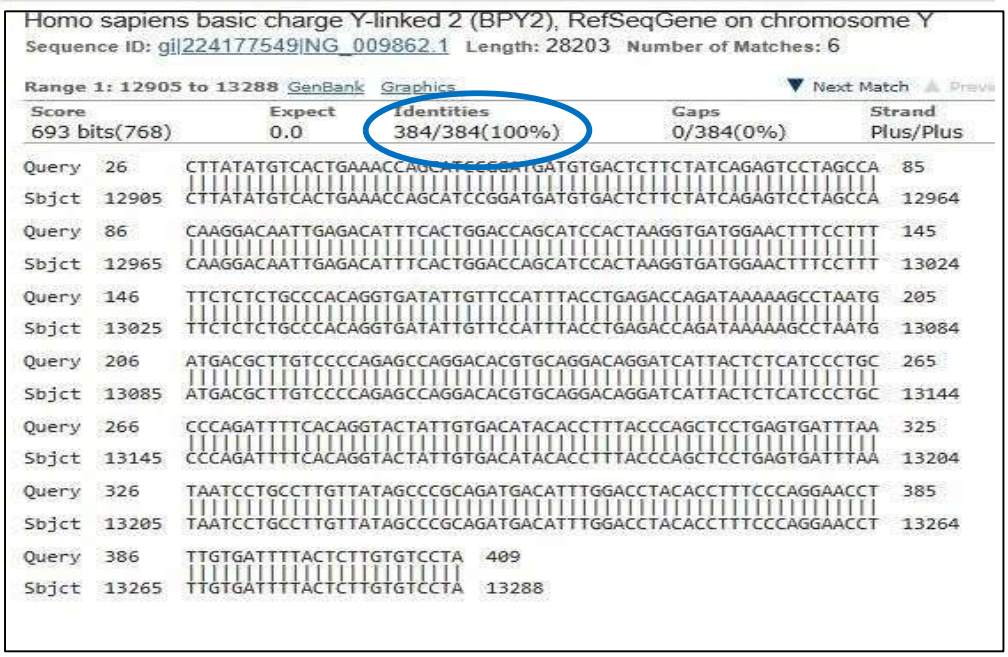

Figure 4: BLAST Search Showing 100% Similarity with the NCBI Reference Sequence

IV. Discussion:

Male infertility is a complex reproductive disorder with multiple genetic, environmental, and lifestyle-related factors contributing to its prevalence [22]. Among genetic causes, Y chromosome microdeletions are recognized as significant contributors, particularly those affecting the AZFc region, which contains multiple genes

essential for spermatogenesis [11]. The *BPY2* gene, located within the AZFc region, has been implicated in spermatogenic failure, and its deletions or sequence variations have been associated with severe oligozoospermia and azoospermia [13]. This study aimed to investigate the genetic variations in Exon4 to Exon8 of *BPY2* using Sanger sequencing in Bangladeshi azoospermic male patients. This study findings showed 100% sequence similarity with the NCBI reference sequence, indicating an absence of mutations or deletions in the sequenced regions of *BPY2* among the study population.

The absence of *BPY2* gene variations in this study contrasts with several previous investigations reporting AZFc deletions as a common cause of male infertility. A study by Hopps et al. (2003) detected Y chromosome microdeletions, including AZFc, in azoospermic and severely oligozoospermic men, supporting the hypothesis that genetic defects in this region can lead to spermatogenic failure [16]. Similarly, Foresta et al. (2005) identified AZFc deletions among men undergoing intracytoplasmic sperm injection (ICSI), reinforcing the role of Y-linked genetic factors in severe male infertility [23]. However, not all studies have reported a direct correlation between AZFc deletions and azoospermia. Ravel et al. (2006) analyzed over 10,000 sperm donor karyotypes and found that some fertile men carried AZFc deletions without evident reproductive impairment, suggesting that not all deletions within this region lead to infertility [24]. This aligns with this stydy findings, as no sequence variations were detected in the *BPY2* exonic regions among the azoospermic cohort. The results also support findings by Pelzman & Hwang (2021), who emphasized the heterogeneity of genetic causes in male infertility and noted that not all Y chromosome microdeletions directly cause azoospermia [25]. Similarly, Boivin et al. (2007) highlighted the importance of distinguishing genetic infertility from cases influenced by environmental or idiopathic factors, which could explain the lack of *BPY2* mutations in the cohort [26].

The absence of *BPY2* gene variations in this study may be attributed to ethnic and geographic differences in genetic susceptibility. Cavkaytar et al. (2012) found that the frequency of Y chromosome microdeletions varied significantly between different populations, suggesting that the genetic basis of male infertility is population-specific [27]. Additionally, Rahman et al. (2016) reported a relatively lower prevalence of genetic causes in Bangladeshi infertile men, compared to global reports, further supporting the hypothesis that environmental, lifestyle, or non-genetic factors could play a more significant role in this population [28]. Studies conducted in China and Turkey also observed regional variations in the prevalence of AZFc deletions, indicating that the genetic landscape of male infertility may differ across populations [27]. Given that 87.5% of the study population had no known exposure history, the likelihood of environmental infertility factors influencing our results is low. However, further research involving larger sample sizes and whole-genome sequencing approaches is required to explore additional genetic variations in non-coding or regulatory regions of *BPY2*.

One of the strengths of the study was the use of Sanger sequencing, which provides high-fidelity DNA analysis and is widely regarded as a gold standard for detecting point mutations and sequence variations [29]. However, this study was limited to Exon4 to Exon5 of *BPY2*, and did not assess regulatory regions, copy number variations (CNVs), or whole-gene deletions, which may also contribute to spermatogenic failure.
Additionally, this study focused exclusively on azoospermic males, and further research should include oligozoospermic and normospermic control groups to provide a broader understanding of *BPY2* gene's functional role in male fertility. Future studies should also employ high-throughput sequencing techniques, such as next-generation sequencing (NGS), to explore novel mutations that might not be detectable through Sanger sequencing [30].

This study reveals no detectable *BPY2* exonic mutations or deletions in Bangladeshi azoospermic men, suggesting *BPY2* variations are not a major contributor to infertility in this population. While AZFc deletions are linked to male infertility, the genetic basis of azoospermia is complex and multifactorial, warranting large-scale studies integrating genetic, environmental, and epigenetic factors.

V. Conclusion:

This study found no *BPY2* exon mutations or deletions in Bangladeshi azoospermic men, suggesting its variations are not a major cause of azoospermia in this population. Given the low prevalence of Y chromosome microdeletions, environmental or idiopathic factors may play a greater role. While Sanger sequencing ensures accuracy, this study was limited to Exon4 and Exon5, excluding potential regulatory or structural variations. Future research should incorporate larger cohorts, whole *BPY2* gene sequencing, and haplogroup analysis to better understand genetic influences on male infertility. Expanding genetic screening is crucial for improving azoospermia diagnosis and treatment.

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