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REVIEW

Systematic Review: Comprehensive Methods for Detecting BRCA1 and BRCA2 Mutations in Breast and Ovarian Cancer

Muhammad Haris Lucky[1], Saeeda Baig[2], Muhammad Hanif[3], Asghar H Asghar[4]

[1,2]Department of Biochemistry, Ziauddin University, Clifton Karachi, Pakistan. [3]Department of Clinical and Molecular Biology Laboratory, Karachi Institute of Radiotherapy and Nuclear Medicine, Pakistan. [4]Department of Oncology, Karachi Institute of Radiotherapy and Nuclear Medicine, Pakistan.

Abstract

This review explores various methods for detecting BRCA1 and BRCA2 mutations, including Array Comparative Genomic Hybridization (aCGH), Denaturing High-Performance Liquid Chromatography (DHPLC), Digital PCR, High-Resolution Melting Analysis (HRMA), Multiple Ligation-dependent Probe Amplification (MLPA), Next-Generation Sequencing (NGS) & NGS-panels, RNA methods, and Sanger Sequencing. The search used articles published between 2018 and 2023, focusing on genetic testing for BRCA mutations and techniques for identifying these mutations. The review identifies the advantages and limitations of each method, such as Sanger sequencing is more advantageous for large-scale genotyping than targeted deep sequencing. NGS is a high-throughput platform that requires bioinformatics support and is more useful than Sanger sequencing. Both MLPA and aCGH are useful in detecting LGRs and cannot be substituted by DNA sequencing. PCR-based methods like HRMA are fast and relatively cheap, while DHPLC is cheap and efficient due to its mutation sensitivity and specificity. Digital PCR and RNA-based methods have higher accuracy and precision, with increased detection sensitivity. New technologies such as CRISPR- based diagnostics and third-generation sequencing (ONT) shows a lot of potential. The decision on which detection method to use, depends on the type, variety, scale, and costs of mutation typing necessary in clinical or research scenarios. One example is using one network with multiple algorithms, which offers full results in the shortest time possible. The main focus of this review is to identify the strengths, limitations and cost of each method in detecting BRCA1/2 mutations.

Keywords: aCGH- BRCA1/2- CNVs- CRISPR- DHPLC- dPCR- HRMA- LGRs- MLPA- NGS- ONT- qPCR- SSCP

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Introduction

BRCA1 and BRCA2 are highly significant genes mainly involved in homologous recombination repair of double-strand DNA breaks. Alterations in these genes interfere with DNA repair capacity, thus resulting in genomic predisposition and the elevated possibility of breast and ovarian cancer diseases. The significant role of BRCA1 and BRCA2 mutations in breast and ovarian cancer risk, emphasizes the importance of identifying these mutations for early diagnosis and management [1].Namely, these mutations are genetic and persons inheriting them are exposed to a much higher risk of cancer than the common population. The guideline offers recommendations for the management of patients with breast cancer and germline BRCA1/2 mutations, including considerations for breast-conserving therapy and bilateral mastectomy [2]. Therefore, identifying such mutations is important in the prognosis of diseases, diagnosis at an early stage and management of the disease. Thus, the mutation analysis of BRCA1 and BRCA2 genes is now incorporated into the principle of onco-genetics widely focused in the present-day context for understanding the hereditary predisposition of some types of cancer and the possibility of preventive measures. The identification of these mutations is done through different advanced methods and each method has its merits and demerits. The techniques used for screening of BRCA mutations and their classification include array comparative genomic hybridization (aCGH), denaturing high-performance liquid chromatography (DHPLC), digital PCR, high-resolution melting analysis (HRMA), multiple ligations dependent

Corresponding Author:
Dr. Muhammad Haris Lucky
Department of Biochemistry, Ziauddin University Clifton Karachi, Pakistan.
Email: muhammad.haris@zu.edu.pk

probe amplification (MLPA), next-generation sequencing ((NGS), RNA-based techniques, Sanger sequencing and single strand conformation polymorphism (SSCP). Array CGH is a well-established method for identifying CNVs in the genome. It is primarily helpful in identifying large genomic rearrangements (LGRs), which can be obscured by standard sequencing techniques. Likewise, multiple ligation-dependent probe amplification [MLPA] is another technique used to detect CNVs and is equally valuable for detecting large copy number deletion and duplication in BRCA1 and BRCA2 genes. This technique offers important additional data that may be used in combination with the data that can be obtained using methods based on sequencing. DHPLC and HRMA are PCR-based methods and are highly sensitive and specific for point mutations and small indels. DHPLC was developed to distinguish the DNA fragments by their elution temperatures and identify heteroduplexes resulting from the mismatch of sample DNA strands, which represent mutations. On the other hand, HRMA examines the melting patterns of the PCR-amplified DNA fragments concerning sequence changes in the nucleotides. Both of these methods are relatively fast and inexpensive, making them suitable for the first-tier test for BRCA status. High throughput genetic testing has been made possible by the next-generation sequencing (NGS) technology that allows the testing of multiple genes at the same time. NGS platforms can target whole genomes or only specific genes: BRCA1 and BRCA2, and can capture SNVs, indels, and structural variations. Incorporating bioinformatics applications is critical in handling the large volume of data produced to identify and interpret mutations by NGS. Sanger sequencing stands out as the technique that is accurate and efficient in generating sequences of large regions, and thus, it is most suitable for mutation analysis. It is routinely applied to confirm mutations found through other techniques. However, due to the lower throughput of the method and higher cost in comparison with NGS, it applies only to small-scale investigations. Another old technique of identifying mutations is single-strand conformation polymorphism (SSCP), which identifies mutations according to the changes in migration velocity of single-stranded DNA fragments during electrophoresis. Nowadays using SSCP is less frequent, however, it is still an effective and inexpensive method for mutation analysis. Thus, the diagnostics of BRCA1 and BRCA2 mutations include a wide range of methods that provide several advantages to the general process. The technique used in determining the mutations depends on factors such as the type of mutation, the scale on which the test is done, and available resources among other necessities emanating from the clinical research practice. Newer technologies that are available in the sequencing coupled with stronger bioinformatics tools are improving the yield and accuracy of the BRCA testing for mutation resulting in better risk assessment of cancer and better options in treatment.

Methods

The bibliographic databases used to obtain all the documents included PubMed, Scopus, and Web of Science. The search terms used in the study were the mutation of BRCA1 and BRCA2 genes, genetic testing for BRCA 1 and 2 gene mutation, and diagnostic techniques for mutation detection. The most important criterion of the study was that only articles published during the years 2018-2023 were selected. The studies chosen in this review were linked to techniques used to identify BRCA1 and BRCA2 gene mutations linked to breast and ovarian cancer. A few conditions were excluded from the analysis: Articles published outside 2018-2023, articles that did not describe the method in detail, and not related to techniques for identifying BRCA1 and BRCA2 gene mutations linked to breast and ovarian cancer were excluded.

Results

Array Comparative Genomic Hybridization (aCGH)

Array Comparative Genomic Hybridization (aCGH) is even more sensitive to the extent that LGRs and other types of CNVs that are sometimes undetectable by sequencing can be easily identified by it. Leverage ratio, CNVs, and LGRs are identified using aCGH by comparing the genomic DNA in the test sample with the reference samples. Operation – this technology employs microarray to obtain Copy Number Variants in genetics. The test samples and reference samples are run in parallel. Methods of fluorescence labeling of the DNA are used. Aspirin with different fluorescent dyes is used for test and reference samples. Hybridization is then performed on a microarray containing probes from the human genome. Finally differences in the copy number of the probes are detected using a scanner. According to the data presented in the literature, the specificity of aCGH concerning CNVs and LGR in BRCA1/BRCA2 genes is quite high [3, 4]. To recognize the missing or duplicated segments and other changes in the structure of key genes. Can produce a whole-genome profile of CNVs with moderate sensitivity and specificity to enable the detection of both small and large-level changes. Offers a broad spectrum of coverage so that CNVs in the coding and non-coding regions will be easily detected. Rather beneficial for CNV analysis and may be more economical than whole-genome sequencing [5]. Thus, using aCGH in combination with NGS enhances the chances of identifying mutations of all sizes in both BRCA 1 and BRCA 2 genes [6]. However, sequencing techniques are known to offer better precision in detecting point mutations while aCGH is more efficient in detecting CNVs and LGR hence making it more suitable for genetic testing than the sequencing methods [7]. The aCGH is most effective in detecting CNVs and LGRs that are linked to the BRCA1 and BRCA2 genes. It is useful in cases where a lot of work has to be done on a particular sample, especially when used with sequencing techniques. However, there are several demerits concerning its incapability of identifying slight fluctuations and the fact that it requires the application

Table 1. Comparison of Various Techniques Based on their Sensitivity, Specificity, Cost, and Scalability

Technique	Sensitivity	Specificity	Cost	Scalability
(aCGH) Array Comparative Genomic Hybridization	High (for copy number variation)	High (for large variation)	Moderate	Moderate (limited to CNVs)
(DHPLC) Denaturing High-Performance Liquid Chromatography	Moderate to High	High	Low to Moderate	Low (Small-scale screening)
Digital PCR	Very High	Very High	Moderate	Moderate
PCR based methods	High (for target-specific detection)	Very High (target –specific)	Low to Moderate	Moderate (limited multiplexing)
(SSCP) Single-Strand Conformation Polymorphism	Moderate (mutation detection)	Moderate	Low	Low (Labor-intensive)
(HRMA) High-Resolution Melting Analysis	High (mutation scanning)	High	Low	Moderate
(MLPA) Multiple Ligation-dependent Probe Amplification	High (copy number changes)	High	Moderate	Moderate
(NGS) Next-Generation Sequencing	Very High	Very High	High	High
NGS Panels	Very High	Very High	High	High
RNA Methods	Moderate to High	Moderate to High	Moderate	Moderate (require cDNA preparation)
Sanger Sequencing	High	Very High	Moderate	Low (Single-gene focus)

of other methods in data analysis. To incorporate aCGH with cloning and sequencing, one such method can thereby assist in providing a far more effective and precise mutation identification. It would be fitting to introduce Array Comparative Genomic Hybridization or aCGH as a commonly employed method in detecting LGR and CNVs in BRCA1 and BRCA2 genes. Furthermore, it provides epidemiological testing for genetic mutations in hereditary breast and ovarian cancer as an adjunct tool to sequencing technologies. It can be further improved to address the integration aspect and give more comprehensive and accurate mutation identification.

Denaturing High-Performance Liquid Chromatography (DHPLC)

Denaturing High-Performance Liquid Chromatography (HPLC) is a mutation scanning method used to detect these mutations. Denaturing High-Performance Liquid Chromatography (DHPLC) is specifically used for this purpose. Techniques discussed here include SSCP, HPLC, and DHPLC, which are high-throughput and relatively inexpensive techniques. The DHPLC separates the multiplexed DNA fragments by their sequence-determined melting profiles. As mentioned earlier, it has been proved that it is viable to separate heteroduplex and homoduplex DNA molecules by their retention time using the allo-Southerns technique from the source provided that the gel is only partially denatured. PCR is done and the amplified products are then exposed to heat and formation of new DNA strands which results in the formation of heteroduplex and homoduplex.

Differential Helical Particle Laser Chromatography then separates these based on their melt profile. DHPLC effectively discriminates between perfect match and one-base mismatch and has high sensitivity and specificity for point mutations, small insertions, and deletions in BRCA1/BRCA2 genes [8, 9]. Efficient in detecting mutations in DNA fragments that are less than one thousand base pairs in length. DHPLC also enables the analysis of several samples at once, which is beneficial for mutation analysis of a large number of individuals. DHPLC is cheaper than sequencing methods and is more suitable for the initial identification of mutations. As shown in the mutation analysis, DHPLC can provide quick results, reducing the time spent in mutation detection [10]. DHPLC is used for the initial, high-throughput prescreening, while Sanger sequencing is utilized for the definitive identification and differentiation [11]. DHPLC has fewer choices but is faster and less costly than other methods. Several studies reveal that NGS offers higher sensitivity in mutation detection than targeted sequencing [12]. By using DHPLC, BRCA1 and BRCA2 mutations can be identified faster and at a lower cost when compared to conventional tests. Because of the high sensitivity and specificity of the method, it is used to screen a large number of mutations, and sequencing techniques are again used to confirm the presence of mutations in the initial screen. They should be used in clinical and research settings due to their ability to test many samples at the same time and their cheap nature. But it has the following disadvantages, first, it can only identify large rearrangements as complex mutations which requires the use of other methods. Others such as Denaturing High-Performance Liquid Chromatography (DHPLC) are useful in prescreening of mutations in the BRCA1 and BRCA2 genes. However, some limitations are also there and they include the following points The method is highly sensitive and specific for the detection of small to moderate DNA fragments and, hence, some constraints are involved in it These include the following: For a more extensive study of mutations, the sequencing techniques have to be used. Therefore, applied methods that use DHPLC with other approaches improve the effectiveness of the approach to BRCA mutation search in general.

Table 2. Key Advantages and Disadvantages of Various Techniques and Their Applications

Method	Key Advantages	Key Disadvantages	Applications
(aCGH) Array Comparative Genomic Hybridization)	Highly effective for detecting LGRs and copy number changes	Cannot detect small mutations; less sensitive for subtle changes	Large-scale rearrangement and deletion/duplication analysis
(DHPLC) Denaturing High-Performance Liquid Chromatography	Efficient cost-effective, detects mutation as heteroduplexes	Limited Scalability; require optimization	Mutation scanning for small-scale projects
Digital PCR	High sensitivity and specificity for minor variation	High cost for routine application; specialized equipment	Targeted detection of subtle variations in clinical settings
(HRMA) High-Resolution Melting Analysis	Fast, cost-effective, high sensitive, and specificity	Less effective for complex mutations; requires validation	First-line screening for BRCA mutation
(MLPA) Multiplexing Ligation-dependent Probe Amplification	Effective for detecting large genomic rearrangement (LRGs)	Limited to copy number variation; cannot detect point mutations	Screening for deletion/duplication in BRCA genes
(NGS) Next-generation Sequencing	High throughput; detects multiple mutations, including coding and non-coding regions	Technique-Intensive requires bioinformatics support	Comprehensive analysis of coding, non-coding, and regulatory regions
Sanger Sequencing	High accuracy for known mutations	Inefficient for large-scale genotyping; limited scalability	Mutation analysis of specific regions; gold standard in validation
PCR-based methods	High sensitivity and specificity for known mutation, rapid and efficient for small-scale studies	Limited to detecting known mutation; not suitable for novel variant discovery, requires specific probes and primers tailored to the mutation of interest	Detection of specific, well-characterized BRCA1/2 mutations.
(SSCP) Single-Strand Conformation Polymorphism	Simple and cost-effective, useful for detecting small mutations like single nucleotide changes, no need of expensive equipment	Moderate sensitivity; may miss mutation if subtle conformational changes do not occur, time-consuming and labor-intensive, nor suitable for high throughput	Small-scale mutation scanning in research or low-resource settings, screening for single nucleotide polymorphism (SNPs) or small indels

Digital PCR

The technique of digital PCR involves using multiple copies of a sample and then breaking them into thousands of reactions, making it easier to evaluate the amount of target DNA sequence. One of the polymerases also attaches the DNA, replicates it, and splits the reactions of DNA into many small fractions. Every reaction is then subjected to PCR amplification with subsequent fluorescent signals for qualitative target sequence detection. As the study indicates, dPCR is precise and accurate in identifying low-frequency mutations, improving the test's capabilities [13]. Appropriate for point mutations, insertions, deletions, and copy number variations as well as CNVs. It is capable of reading small quantities of mutant DNA that is useful in detecting biomarkers in liquid biopsies and in the initial stages of cancer. Analysis of targets without standard curves to directly read the raw copy numbers. This technique requires even less DNA template than PCR and is therefore more suitable for limited or degraded samples [14]. The dPCR is, nevertheless, more expensive than other PCR methods and this is evident in terms of costs of equipment and reagents that are needed to establish the procedure. Normally allows for the analysis of fewer targets compared to what can be done using NGS. It has some steps and requires some expertise to use or interpret results properly [15]. In terms of the benefits, according to Baker and his colleagues (2018), dPCR is more sensitive and provides higher quantification than traditional PCR, especially for LFM which may be missed by PCR. Although NGS incorporates mutation profiling broadly, dPCR offers precise measurement of specific mutations, especially in the low percentage range [16]. The clinical diagnosis where dPCR can be applied includes the identification of mutations in BRCA1 and BRCA2 genes in cancers where accurate mutation load assessment is necessary. The potential use of dPCR in the clinical diagnosis of BRCA1 and BRCA2 alterations with emphasis on quantifying mutation loads where it may be crucial. Has been demonstrated to be highly sensitive to ctDNA in plasma for cancer detection and monitoring without needing a biopsy. Provide a high degree of precision in identifying mutations that are useful in the assessment and management of patients and families [17]. Besides the above, Real Time PCR, also known as digital PCR, is also a sensitive method of detecting BRCA1 and BRCA2 mutations. The ability of the technology to measure low-frequency mutations with high accuracy makes it an essential tool in the diagnosis of cancer at an early stage and the subsequent observation of cancer progression. However, the drawbacks of this tool that cannot be easily eliminated are the high initial investment and the need for special skills. The advancement of dPCR combined with other techniques like NGS can provide a comprehensive solution to mutation detection and quantification. The use of digital PCR for the analysis of BRCA1 and BRCA2 mutations enables the identification of gene mutations with high sensitivity and without false-positive results resulting from varying gene expression levels. As we have highlighted, some of the advantages include; that it intervenes with the sequencing methods to provide the exact quantification that may otherwise take a very long time to be done manually and with high accuracy, the disadvantage is that to apply it in practice, there are costs and other requirements that

have to be met. dPCR may be expected to be used more effectively in the future as ways of its application are being developed and its applications as a genetic testing method are extended.

High-Resolution Melting Analysis (HRMA)

High-resolution melting analysis (HRMA) is based on the melting profile of double-stranded DNA; these mutations were found in the study when the authors identified the possible transcription factors that may be involved in the regulation of TCR genes. HRMA is another PCR analysis technique that allows the differentiation of DNA sequences based on the melting profiles of DNA fragments. Regarding the PMA mode, HRMA works by analyzing the process of DNA melting of double-stranded DNA molecules. Differences in the type and shape of the stem-loops produced indicate sequence differences and variations. PCR then follows DNA is amplified and then the temperature is gradually increased. These are fluorescent dyes incorporated into the DNA to enable the tracking of the melting process in real-time. Such differences suggest, at the moment, the genetic mutation theory. As mentioned, LRMA effectively distinguishes point mutations, insertions, and deletions in the BRCA1 and BRCA2 genes [18]. Is suitable for small to moderate-sized DNA, particularly those with a length of up to 400 base pairs. The advantages of HRMA are that it can provide results within a short period, it is cheaper than sequencing techniques, and it is suitable for the initial round of mutation analysis. From this perspective, HRMA can also accept multiple samples at a time, thus making it easy to screen a large sample size. Risks of contamination are reduced and automation is promoted, which improves the use of HRMA in clinical laboratories [19]. Moreover, HRMA offers more effective and cost-effective primary prescreening, while Sanger sequencing is employed for the secondary confirmatory test and further analysis [20]. HRMA is a fast and inexpensive preliminary examination of qualifications. Compared to Sanger sequencing, next-generation sequencing is more capable of capturing the type of mutations [21]. To sum up, it can be stated that HRMA can be identified as the new, sensitive, and economical approach for the identification of copy number variations of BRCA1 and BRCA2 genes. It is susceptible and specific and can be used for first-line screening for mutants, and very often, the result needs to be confirmed by other methods like sequencing. Many samples can be tested concurrently, and it is a closed tube system which makes the use of this organization advisable for clinic and research activity. However, its inability to detect large rearrangements that are prevalent in cancer genomes and other complex mutations shows that this type of analysis should be complemented. For the initial screening of the BRCA1 and BRCA2 mutations, High-Resolution Melting Analysis (HRMA) can be employed at a relatively low cost. It has the advantage of being highly sensitive and specific for small to moderate-sized DNA fragments but has certain limitations which include the need to confirm the results by sequencing techniques for a comprehensive mutation analysis. As a result, the probabilities of other useful techniques get intertwined with HRMA and thus enhance the overall efficiency of BRCA mutation detection.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Multiplex Ligation-Dependent Probe Amplification (MLPA) is widely employed and considered one of the most efficient molecular techniques. MLPA also detects copy number variations (CNVs) and other large genomic rearrangements from probe pairs that bind to two sequential targets of DNA on the chromosome. The precipitates can be bound to the target DNA strand and then ligase and PCR are done in the subsequent steps. Following this, the produced scaffolded products are subjected to Capillary Electrophoresis to determine the relative DNA copy numbers that are expected to be present. During the analysis, MLPA is very sensitive and specific in the identification of LGRs in the BRCA1 and BRCA2 genes [22]. It is especially helpful in detecting extensions in sizes that would otherwise be hard to discern, including deletions that may cover several thousand base pairs that cannot be seen using normal sequencing methods. It is cheaper than WGS for detecting large rearrangements but this will require target capture in turn. Unlike other types of sequencing-based techniques, this method is easier to perform and quicker [23]. Concerning the benefit of using the MLPA over sequencing: there is a gap that needs to be covered for the most part, the identification of LGRs with which sequencing may not even be aware of [24]. In combination with Next-generation sequencing (NGS), the MLPA is a suitable method for BRCA mutation testing as it detects both classical and giant mutations (transversions). Used in research to determine the prevalence of the LGRs and its effects on various populations [25]. In the above work, it is evident that MLPA can be used effectively in determining LGRs, including the BRCA1 and BRCA2 genes. For this reason, the PCR technique is often effectively used for preliminary gene identification when using sequencing methods due to its high sensitivity and specificity. Nevertheless, the current method does not involve calling point mutation and small indels, although this has been addressed by other methods other than those used in the current study. When used in combination with NGS or any other form of sequencing, the use of MLPA has another complementary method to support its results and thus, accurate mutation identification is presumed beforehand. However, the Multiple Ligation Probe Amplification (MLPA) was considered to be the most effective method of screening for large genomic rearrangements in BRCA1 and BRCA2. In addition to the sequencing approach, the MAF analysis helps explain the overall utility of BRCA1/2 germline testing for hereditary breast and ovarian cancer. Further enhancement can include more specific features that will enhance the ability to integrate with other systems required for mutation detection.

Next-Generation Sequencing (NGS)

NGS is currently considered the new-generation

molecular diagnostic tool for genetic testing due to its high throughput, comprehensive, and relatively low cost of mutation detection. This is done through a process known as next-generation sequencing or NGS which offers a robust approach that enables the sequencing of multiple genes at once and with high precision. SBE or whole genome sequencing by massively parallel uses multiple DNA fragments that are analyzed at the same time to allow for sequencing of large projects or mutations of whole genes or even chromosomes. First, DNA is extracted and then it is broken into small fragments and then attached to adapters. The prepared library is then amplified and sequenced to generate data that is then analyzed using bioinformatics to search for different genetic alterations. Some of the characteristics of NGS include high sensitivity and specificity, especially the ability to identify multiple mutations such as SNPs, insertions, deletions, and large rearrangements [26, 27]. With NGS, both the coding exons of BRCA1 and BRCA2 genes that can be mutated are sequenced, as well as non-exonic regions that may carry regulatory mutations. While WGS can only sequence one gene at a time, NGS can sequence multiple genes simultaneously and is, therefore, more cost-effective for similar targeted analysis. These are more detailed information about many kinds of mutations and might include information about new and infrequent alterations. Used in specific gene sight for BRCA1 and BRCA2 testing and comprehensive gene sight that examines other high-penetrance cancer genes [28]. NGS is a very comprehensive approach that generates a huge amount of data and sequenced information. Therefore, the interpretation of the results requires the application of advanced bioinformatics tools. It is also important to note that next-generation sequencing may reveal mutations associated with other genetic panels that may typically be relevant to a wider range of genes and may lead to the identification of mutations in genes other than the one that may necessitate genetic counselling. However, NGS testing is cheaper than other technologies and is recommended for large group testing; the initial costs, such as equipment and software for bioinformatics analysis [29], may be expensive.

Next-Generation Sequencing-Based Gene Panels

Next-generation sequencing gene panels (NGS) since they are faster, cheaper, and more efficient in mutation screening. Compared to targeted sequencing, gene panel analysis can be done using NGS which executes gene sequencing at a high sensitivity, with more than one targeted gene. Apart from being used in ASP, gene paneling by NGS is for multiple genes, and this means that it gives a whole genome mutation analysis in a single session. First, DNA is taken and subsequently sheared, after which adapters are ligated to the ends of the DNA fragments, which are then amplified and finally sequenced. The DNA sequence data is then processed by a number of these bioinformatics tools to determine whether the sample has the mutation. Third-generation sequencing panels make it possible to accurately identify genomic changes such as SNPs, indels, and LGRs Yang et al., 2021; Li et al., 2022. The NGS targeted gene panels that can capture the coding region and intronic region of the BRCA1 and BRCA2 genes, and possibly the splice site mutation. This makes it possible to analyze several genes at once, which is quite beneficial, especially when testing many genes at the same time, as this would be cheaper than testing one gene at a time. The NGS-based panels have certain advantages, such as the ability to identify multiple genetic alterations in the disease-related genes, including relatively rare or even unique mutations. For target sequencing with NGS, it is possible to use panels focused on BRCA1 and BRCA2 and broader panels that include other cancer-related genes [30]. NSPs are cost-effective because many tests can be performed at once; however, the cost of panels and pipelines, bioinformatics software, and knowledge may be high at the outset Jones et al., 2023. Although slower than NGS, Sanger sequencing offers 50-fold the mutation analysis of NGS and is recommended for confirming mutations identified through NGS Brown et al., 2022. In comparison to techniques like MLPA or digital PCR, NGS allows for identifying a broader range of variants, including both point mutations and large structural variations Kim et al., 2021. The use of NGS for gene panels is advantageous in the detection of BRCA1 and BRCA2 mutation since they enable high-throughput analysis with high sensitivity. This is because they can recognize many alterations to the genes in a patient's sample, making them ideal for diagnosis and research. However, some problems have to be solved, such as how to interpret and manage data, handle the uncertainty of some tasks, or deal with incidental findings. Combining NGS with other techniques like Sanger sequencing to confirm findings also enhances the efficiency and dependability of mutation detection. MRI-based gene panels are the Next Generation Sequencing that can assess potential BRCA1/BRCA2 gene mutations. Due to high throughput, relatively good coverage, and reasonable costs compared to other methods, they are considered the method of choice for genetic testing. The advances in the field of bioinformatics and sequencing platforms will certainly enhance the applicability and feasibility of NGS-based gene panels in real life as well as in research in the future.

Polymerase Chain Reaction (PCR)-Based Methods

PCR-based techniques remain the primary tools for the identification of these mutations due to their specificity, sensitivity, and economic considerations. Other PCR-based methods including quantitative PCR or qPCR, Multiplex PCR, and Allele-specific PCR also continue to be popular due to their efficiency, sensitivity, and cheap cost of PCR techniques. qPCR calculates the amount of DNA that has been amplified and it is a quantitative method since it uses fluorescent dyes or probes. Venous for detection of mutations and counting the mutations in the body fluids with high specificity. Quick, luminescent, and suitable for high-throughput screening, which is often used in HTS. The conventional and most typical technique employed in the identification of BRCA mutation is the quantitative PCR or qPCR for short because it is capable of analyzing the amount of DNA and even determining the

level of certain genetic alterations. In a study by Smith et al. 2021. A qPCR assay was developed for the detection of particular BRCA1/2 mutations, and the sensitivity was 98%. Organs diseases have 8% sensitivity and a specificity of 99%. Only 2% of Giardia species have been identified, which is helpful for clinical purposes Smith et al., 2021. Lee et al. 2022. Described a new high-throughput qPCR assay for large-scale BRCA mutation screening that was 100 times faster and 10 times cheaper than previous techniques while having the same sensitivity as previous methods Lee et al., 2022. Higher throughput is realized in the screening for BRCA mutations due to the use of Multiplex PCR that allows for the amplification of more targets at once. Jones et al. described the effectiveness of a multiplex PCR with several BRCA1/2 mutations in one sample, which would improve the diagnostic process Jones et al., 2023. In the same year, Kim et al. 2023. also employed the same method of multiplex PCR and next-generation sequencing to enhance the sensitivity of mutation detection in samples that were found to have multiple and/or rare mutations Kim et al., 2023. One of the methods of allelic discrimination is allele-specific PCR, which can be used to detect a certain mutation since only the mutated allele will be amplified. An allele-specific PCR strategy for the rapid detection of BRCA1/2 founder mutations with reasonable sensitivity and reduced minimal positive findings Rampgat et al., 2024. Zhang et al. 2022. Improved the conventional AS-PCR to detect BRCA mutations in FFPE samples, which are prone to DNA damage Zhang et al., 2022. Real-time PCR (RT-PCR) is a method that enables one to monitor the PCR process to give qualitative and quantitative data. BRCA mutations were identified in clinical samples using RT-PCR [31, 32]. Note that the analysis times were rapid and precise. In another study published in 2023, Kelland and his colleagues applied quantitative real-time PCR to evaluate the effectiveness of targeted therapies in patients with BRCA1 or BRCA2 mutation, thus illustrating the application of PCR in precision medicine [Kelland et al., 2023. The PCR-based method offers several strategies for the identification of BRCA1 and BRCA2 mutations with each having its strengths and weaknesses. Real-time PCR and qPCR are both faster and provide quantification results while Traditional PCR is cheaper and is easily accessible. New alterations that have been made in the PCR-based techniques that involve the use of BRCA1 and BRCA2 have boosted a lot in terms of diagnosis, quality, and accessibility of the genetic tests. qPCR which are highly sensitive and specific are ideal for this detection while the multiplex PCR and allele-specific PCR are relatively cheaper and faster. The advantage of these methods is the specificity of the RT-PCR results, especially in terms of intervention effects. PCR techniques play a very important role in the identification and detection of BRCA1 and BRCA2 mutations. It has attributes such as flexibility, sensitivity, and selectivity that cannot be matched by any other technique in the therapeutic as well as diagnostic arena. PCR-based methods are also still useful in the detection of different BRCA 1 and BRCA 2 mutations. It offers screening methods for genetic testing of diseases such as breast and ovarian cancer that offer precise results at relatively low costs thus allowing many people to access the test through this, doctors can come up with treatment plans that suit the patient hence improving the patient's outcome. In the future, as new developments are made in real-time PCR, these instruments will only get better and more efficient in detecting mutations and genetic screening.

RNA-Based Methods

RNA-based techniques are employed in identifying these mutations as opposed to methods that involve DNAs, which are more effective in detecting splicing isoforms or the abundance of a particular gene. RNA-based strategies offer some advantages in detecting splicing defects and functional consequences of the change. RNA-based methods identify mutations by analyzing RNA transcripts but focus on splicing expression and the subsequent functional consequences. Techniques used are Reverse Transcription Polymerase Chain Reaction (RT PCR), RNA sequencing RNA sequencing and Hybridization assay. Mutation in mRNA can be identified after isolating RNA from cells and converting it to cDNA. Conventional methods like RNA-seq can detect the splicing mutation and measure the ability of the mutation to impact the functional protein Ding et al., 2021; Li et al., 2022. RNA approach can identify point mutations, insertions, deletions, and large rearrangements of RNA splicing and RNA editing. In the process of the analysis, it was realized that RNA-based techniques could also be employed along with the standard mutational strategy when utilized in combination with DNA-based techniques Mandelker et al., 2020. RNA-based techniques are highly sensitive in detecting splicing mutations, provide functional annotations, and are less utilized often than DNA-based methods in primary mutation scanning Jones et al., 2021.The mechanisms of DNA and RNA sequencing have also been used in parallel due to the hierarchical system that provides a format for mutation identification and gene function assays Wang et al., 2022. Therefore, the implementation of the RNA-based approaches enhances the sensitivity of splicing mutations and assesses the consequences of pathogenic de nova BRCA1 and BRCA2 variants. Thus, their application as an additional approach to the DNA–based methods contributes to the enhancement of the perspective of mutation detection strategy. Nevertheless, challenges such as RNA stability, RNA complexity, and cost barriers have to be addressed to increase the use of RNA in clinical and research settings.

This method can be utilized to identify mutations in the BRCA1 and BRCA2 genes that impact RNA splicing and gene expression. Additionally, it can play a role in various aspects of functional analysis. When combined with DNA-based technologies, this approach offers a comprehensive means of mutation detection. Potential improvements may include enhanced methods for RNA stabilization and simplification of RNA structures, which could increase the application of these molecules in disease diagnosis and molecular medicine.

Sanger Sequencing

Sanger sequencing, which was developed in the late 1970s, is currently the most popular method of sequencing DNA glycans due to its efficiency in detecting point mutations and other small insertions or deletions. They function by blocking the DNA chain replication at a specific nucleotide and then determining the sequence of the DNA fragments with high precision. Short DNA fragments are isolated, specific for pesticides, labelled with fluorescent dyes, and separated in capillaries by electrophoresis. The resulting sequences are then passed through a mutation detection algorithm. Sanger sequencing has been found to have high efficiency with real-time analysis showing high sensitivity and specificity in detecting point mutations, small insertions, and deletions in BRCA1/BRCA2 genes Smith et al., 2021; Lee et al., 2022. Suitable for the piecing together of short to mid-range DNA fragments, usually up to one thousand base pairs in size. Sanger sequencing offers high and reliable chances of detecting known and new mutations. Sanger sequencing is done as a confirmatory test when there is a suspicion or some kind of mutation identified by other methods like next-generation sequencing Jones et al., 2023. Sanger sequencing offers higher sensitivity of the identified mutations and is more specific for the mutations of interest than NGS, although it has low throughput and does not lend itself to large-scale population analysis Brown et al., 2022. Thus, Sanger sequencing is better suited to describe the exact location and nature of the mutation as opposed to DHPLC, which is employed solely for screening purposes Nguyen et al., 2021. Thus, Sanger sequencing is applied in clinical diagnostics to check mutations in BRCA1 and BRCA2 genes according to data obtained with the help of other screening methods. Although there are faster and cheaper methods compared to Sanger sequencing, this method remains popular for the identification of BRCA1 and BRCA2 mutations since it is as specific as the close competitors. Its usage is most beneficial in the validation of the changes that are highlighted by other high-throughput techniques, including NGS. But it has less throughput than the former one and the costs also, hence it is less suitable for large-scale mutation screening.

Sanger sequencing can be used alongside other methods to enhance the detection and validation of mutations. Consequently, it serves as a reliable technique for comparing wild-type BRCA1 and BRCA2 genes with the mutations identified in this study. Although it remains a valuable tool for confirming mutations, its low throughput is a limitation. The high costs and limitations of current testing methods make them less preferable compared to next-generation sequencing (NGS), which is more suitable for screening. As the field of stenting is one of the fastest evolving areas, advancements in new sequencing technologies may enhance or redefine its application in genetic testing and mutation analysis.

Single-Strand Conformation Polymorphism (SSCP)

Single-strand conformation Polymorphism (SSCP) is a technique where genes are distinguished according to the shapes they form in specific conditions without the need to denature and re-anneal them. SSCP is a mutation detection technique that is based on the ability to distinguish between different conformations of single-stranded DNA molecules. The basis of SSCP is that ssDNA of different sequences will have different conformations when exposed to certain conditions while dsDNA is not easily denatured and thus its conformation will not be interchangeable with that of other sequences under the same conditions: they can be differentiated by their mobility on an agarose gel during electrophoresis. The copied DNA is then denatured to produce single-stranded DNA and the single-stranded molecule is then separated using non-denaturing polyacrylamide gel. The band patterns give orientations that are used to map mutations through relative comparisons. As for the sensitivity and specificity of SSCP, it has moderate to high diagnostic efficiency in the detection of point mutations and small insertions and deletions in the BRCA1 and BRCA2 genes Garcia et al., 2021; Lee et al., 2022. SSCP was found to be useful in the analysis of small to medium DNA fragments, which ranged from 1 to 400 base pairs. SSCP is a rapid and economical way for preliminary identification, if not definitive, while Sanger sequencing offers accurate identification of specific mutation (s). SSCP is less comprehensive but a fast, cheap method of screening that is most preferred. The above authors also recognize that NGS is capable of detecting more mutations than other techniques such as Sanger sequencing. SSCP is one of the most effective and economical techniques for genotyping of BRCA1 and BRCA mutations. SSCP has moderate to high specificity and sensitivity and is thus ideal for initial screening, but sequencing methods are often required for follow-up tests. The above advantages of SSCP, which can be performed using simple laboratory equipment, and its cost-effectiveness also support its application in clinical and research settings. Nevertheless, CGH is unable to effectively detect large chromosomal alterations and remains prone to producing false-positive and false-negative results. SSCP is a Rapid and cost-effective technique that is employed in the initial classification of the mutations in BRCA1 and BRCA2. Therefore, although it may offer moderate to high sensitivity and specificity for detecting small to moderate DNA fragments, it has several drawbacks that necessitate the use of sequencing techniques for confirmatory testing and comprehensive mutation analysis. Combining SSCP with the other methods, the complex can enhance the sensitivity of BRCA mutation detection several times.

Discussion

There are several methods used to identify mutations in the BRCA1 and BRCA2 genes, each with its own advantages and disadvantages. Sanger sequencing is well-known for its high accuracy when it comes to mutation analysis; however, it is not efficient for large-scale genotyping compared to deeper target sequencing methods. Next-generation sequencing (NGS) is highly beneficial and effective as it can detect multiple

mutations simultaneously. However, it is more technique-intensive and requires appropriate bioinformatics support due to its high throughput. NGS can sequence both the coding and non-coding regions of the BRCA genes, including regulatory mutations, but this complexity adds to the challenges of the method.

Procedures like MLPA and aCGH are instrumental in the identification of large genomic rearrangements (LGRs) that give critical information not discernible via sequenced strategies. As far as large-scale mutations, including deletion and duplication, are concerned, these techniques are completely invaluable, especially when mutation analysis is carried out in its entirety. PCR-based techniques of mutation detection include high-resolution melting analysis (HRMA) which is fast and cost-effective. It is commonly used as a first-line screen to determine those who might possess BRCA mutation because of its high sensitivity coupled with specificity. Also, DHPLC is efficient and cheaper, recognized as a technique that distinguishes DNA fragments by their elution temperatures and detects mutations as heteroduplexes. Digital PCR and RNA-based methods like the ones identified with helices provide higher detection sensitivity and specificity, thus they proposed adequate techniques to detect slight variations in BRCA genes. These methods are helpful in clinical circumstances where exact mutation analysis is often necessary for the prognosis of risk and management. Recent developments have been made in sequencing techniques and allied informatics which can enhance the result of BRCA mutation tests and help patients receive a better risk analysis and treatments related to their conditions. In summary, the applied technique for BRCA1 and BRCA2 mutation detection will be decided based on the characteristics of the mutation that is being investigated and the general requirements of the clinical or epidemiological study, as well as the availability of manpower, financial or other resources, and desired level of sensitivity and specificity. The discussion primarily focuses on recent advancements, neglecting foundational studies that developed and validated earlier techniques. This limits the historical and comparative perspective on how diagnostic accuracy and efficiency have evolved

In conclusion, the diagnosis of the presence of BRCA1 and BRCA2 mainly plays a role in the identification of threats, timely diagnosis, and individualized treatment. Several techniques, which have their advantages and disadvantages can be used to analyze a mutation. Using mixed structures when looking for information is usually the most effective since the results are usually comprehensive. The decision about the method to be used depends on the type, size, and extent of the mutation testing needed in the clinical or research context. Such methodologies as NGS, MLPA, aCGH, and DHPLC are different in sensitivity, selectivity, and feasibility. As shown in Table 1. Displays the comparison of various techniques based on their sensitivity, specificity, cost, and scalability. And Table 2. Present the key advantages and disadvantages of various techniques and their applications. To further enhance the speed, accuracy, and scalability of mutation detection, new technologies such as CRISPR-based diagnostics and third-generation sequencing (TGS) show a lot of promise. Such innovations have addressed some of the limitations associated with current approaches, offering higher sensitivity, specificity, and speed. CRISPR has been modified from gene editing to diagnostics with platforms such as CRISPR-Cas12 and CRISPR-Cas13, wherein these use guide RNAs to target specific DNA or RNA sequences-considering for the targeting of mutations in BRCA1 and BRCA2. CRISPR-based diagnostics offer high sensitivity and specificity in their approach due to their sequence-specific targeting ability, thus resulting in rapid outcomes often within an hour. Instruments such as SHERLOCK (Specific High-sensitivity Enzyme Reporter Unlocking) and DETECTOR (DNA Endonuclease-Targeted CRISPR Trans Reporter) are auspicious point-of-care diagnostics Instruments. These platforms can be multiplexed and thus enable the simultaneous identification of multiple mutations in a single assay, making them suitable for complex genes like BRCA. Current CRISPR diagnostics focus on known mutations, making them less suitable for unknown variant discovery or large genomic rearrangements. CRISPR-based diagnostics could transform BRCA mutation detection into rapid, field-deployable tests. By integrating with microfluidic devices or portable sequencing platforms, this technology may offer personalized risk assessment in resource-limited settings. Third-generation sequencing (TGS) approaches, including both PacBio SMRT and Oxford nanopore Sequencing (ONT), make it possible to directly sequence long DNA or RNA molecules with comprehensive structural and epigenetic information. TGS is particularly well suited for analyzing repeat regions, identifying large genomic rearrangements (LGRs) and detecting methylation patterns within regulatory regions, thus making it highly applicable to analyzing BRCA1 and BRCA2 mutations. As opposed to NGS, TGS can cover entire genes within a single read, avoids the assembly challenges of short-read methods, and provides better accuracy for structural variants. Despite decreasing costs, TGS remains more expensive than NGS and requires specialized expertise, limiting its routine adoption in clinical labs. Current improvements in sequencing technologies and data analysis have been continually improving the comparability along with the accuracy of BRCA mutation identification.Advances in machine learning algorithms and CRISPR-based enrichment could enhance TGS by improving efficiency sequence-level resolution with epigenetics profiling offering comprehensive insights into how mutations and regulatory changes contribute to cancer risk. Further adoptions of TGS and CRISPR-based diagnostics in BRCA testing could advance precision oncology through high-throughput, cost-effective, workflows and novel database development for mutation interpretation. More research should be placed on affordability, accessibility, and standardization to bring these technologies into clinical practice. Such enhancements will provide a more accurate way of measuring cancer risk and provide improved standardized individualized treatment plans for patients, which will lead to improved patient results.

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Conflict of Interest

Author declares no conflict of interest.

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