Examination of Acute Glomerulonephritis After Streptococcus (GNAPS) With Primer of pyrogenic exotoxin B (speB) Gene of Streptococcus pyogenes Bacteria

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Abstract: GNAPS is glomerular damage due to poorly managed Streptococcus pyogenes infection. The damage is dominated by toxins produced by S.pyogenes, namely streptopain or SpeB. Laboratory examination serves to diagnose S.pyogenes infection. The discovery of specific biomarkers is a priority to improve the quality of laboratory services to prevent and accelerate treatment. The design of specific primers is needed in the molecular examination of PCR method. This research used literature study from NCBI genebank to obtain SpeB gene sequence with access number L26148.1. The gene sequence was analyzed using Primer3Plus to determine candidate primers, then identified using in silico PCR amplification to determine the number of amplicons and visualization on gel electrophoresis. The results of the gene are specific to the S. pyogenes species, the homology level of the gene in the group of species tested by BLAST also shows 100%. These primers include forward primer F5' GGTGCTGACGGACGTAACTT 3' and reverse primer R3' TGCCTACAACAGCACTTTGG 5'.The primer design was able to amplify the SpeB gene region with an amplicon size of 151. These primers can be used in the examination of GNAPS by PCR method so that it is earlier to be treated so that it will reduce the mortality rate due to complications of GNAPS

Keywords: *Streptococcus pyogenes*, primer PCR, biomarker, *in silico*, GNAPS.

INTRODUCTION

Glomerular acute post-streptococcal nephritis (GNAPS) is an inflammatory disease of the glomerulus in the kidneys caused by infection with Group A Beta hemolytic Streptococcus bacteria, this infection causes the kidneys to experience decreased function (Pardede, 2009, Skrzypczyk, 2021). GNAPS occurs as a result of upper respiratory tract infections and several other skin infections that are not handled properly, epidemiologically GNAPS is experienced by 20% of patients with impetigo infections, 19% of pharyngitis patients and <2% of nephritogenic patients. This infection affects all

people, but is more common in children aged 5 - 10 years and low economic status is also closely related to the incidence of GNAPS in Indonesia. GNAPS

Streptococcus pyogenes is a coccus-shaped bacterium, arranged in rows, measuring 0.5 - 1.0 µm, Gram Positive and is β hemolysis if cultured on media containing erythrocytes. These bacteria is capable in producing hyaluronidase, streptokinase, and hemolysin enzymes that are destructive to body cells. Streptococcus pyogenes also produces toxins such as streptolysin O, streptolysin S, protein M and Streptococcal pyrogenic exotoxin there are 3 types namely SpeA, SpeB and SpeC. SpeA has a very strong superantigen. This means that SpeA can nonspecifically activate some T cells, leading to a massive release of cytokines. This can cause toxic shock syndrome and severe inflammatory symptoms and can inhibit phagocytosis while SpeB is a cysteine protease that breaks down host proteins. It helps bacteria spread through tissues by damaging the structure of host tissues. SpeC has the same properties as SpeA (Deacy et al, 2021).

SpeB has great potential in damaging glomerular cells into glomerulonephritis (Balasubramainan & Marks, 2017). SpeB is the most abundant protein secreted in S. pyogenes culture supernatants and can be released from bacteria from extracellular vesicles (Ni et al, 2023). SpeB deserves special attention because it is a major virulence factor capable of breaking down host proteins such as gasdermin-A (GSDMA) found on epithelial cells, triggering inflammation by mediating the cleavage of the cytokine precursor interleukin-1 beta (IL-1β), inhibiting a number of complement factors such as C2, C3b, C3, C4, C5 that promote host immune evasion, and interferes with adaptive immunity by catalyzing the cleavage and degradation of host immunoglobulins to enhance immune system evasion SpeB It is also evident that cysteine protease end products are well conserved in natural populations of S. pyogenes, providing additional evidence that cysteine protease end products well conserved in natural populations of S. pyogenes. pyogenes, providing additional evidence that these enzymes are involved in host-parasite interactions, and suggesting that proteases play a role in bacterial spreading, colonization, and invasion, and inhibition of wound healing (Kapur et all, 1993).

Laboratory tests have been developed such as microbiological tests, serological tests and molecular tests to detect S.pyogenes bacteria. Sample culture tests are considered the gold standard of S.pyogenes examination, but have limitations in the length of time required for examination and are

prone to contamination (Lagier et al, 2015). Serology test is one of the methods often used in laboratories in the detection of S.pyogenes toxin, but it has not been able to quickly detect antibodies released due to the small quantity. PCR is one of the nucleic acid amplification tests that can detect genes that are sustainable, virulent or specific toxins in a microorganism. The SpcB gene from S.pyogenes bacteria produces a toxin in the form of cysteine protease starting from the time the bacteria infect because it is the main defense system for cell division in the host. This PCR method examination has high sensitivity and specificity, so it is recommended to be used alternative method for examination with diagnostic purposes (Lee et al, 2018).

The discovery of biomarkers has been a well-researched trend over the past decade. Appropriate biomarkers help improve examination methods that are more specific to the target (Purba & Manurung, 2018). Detection of Streptococcus pyogenes bacteria with the SpeB toxin gene can be found. Primer design is important and very decisive in the PCR method. Specific primers are made from DNA sequences that have been sequenced. Primers can be designed to amplify specific genes, which provides a greater opportunity to provide a greater effect to get good PCR results according to the target (Etchica et al, 2019).

The purpose of this study was to obtain a primer design that can be used to limit the region to be amplified against the speB gene that was designed in silico. So that the designed primers can be used in the amplification process using PCR, which can later be used as a biomarker primer for rapid detection of Streptococcus pyogenes infection.

METHOD

Literature studies were used to obtain information on the pathogenic factors of S. pyogenes bacteria, especially the toxins produced. The specific toxin was searched for its gene sequence from the NCBI genebank URL http://www.ncbi.nlm.nih.gov with access number L26148.1 (Streptococcus pyogenes pyrogenic exotoxin B (speB) gene) (Figure 1). This gene sequence was used as the basis for the primers and their 3D model (Figure 2).

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GTTGTCAGTGTCAACTAACCGTGTTATTGTCTATTACCATTCATGGTATCAGCGACATCGTATGATAACCATG
CGATTCAGCTAAGTAAGGAGGTGTGTCCAATGTACCGTTAAAAGCAAATGCAGTAGATTAACTTATTTTGAAA
GAGGTATAAAAAAAATGAATAAAAAGAAATTAGGTATCAGATTATTAAGTCTTTTAGCATTAGGTGGATTTGT
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TCTTGCTAACCCAGTATTTGCCGATCAAAACTTTGCTCGTAACGAAAAAGAAGCAAAAGATAGCGCTATCACA
TTTATCCAAAAATCAGCAGCTATCAAAGCAGGTGCACGAAGCGCAGAAGATATTAAGCTTGACAAAGTTAACT
TAGGTGGAGAACTTTCTGGCTCTAATATGTATGTTTACAATATTTCTACTGGAGGATTTGTTATCGTTTCAGG
AGATAAACGTTCTCCAGAAATTCTAGGATACTCTACCAGCGGATCATTTGACGCTAACGGTAAAGAAAACATT
GCTTCCTTCATGGAAAGTTATGTCGAACAAATCAAAGAAAATAAAAAATTAGACACTACTTATGCTGGTACCG
CTGAGATTAAACAACCAGTTGTTAAATCTCCTTGATTCAAAAGGCATTCATTACAATCAAGGTAACCCTTACA
ACCTATTGACCTGTTATTGAAAAAGTAAAACCAGGTGAACAATCTTTTGTAGGTCAACATGCAGCTACAGGAT
GTGTTGCTACTGCAACTGCTCAAATTATGAAATATCATAATTACCCTAACAAAGGGTTGAAAGACTACACTTA
CACACTAAGCTCAAATAACCCATATTTCAACCATCCTAAGAACTTGTTTGCAGCTATCTCTACTAGACAATAC
AACTGGAACAACATCCTACCTACTTATAGCGGAAGAGAATCTAACGTTCAAAAAATGGCGATTTCAGAATTGA
TGGCTGATGTTGGTATTTCAGTAGACATGGATTATGGTCCATCTAGTGGTTCTGCAGGTAGCTCTCGTGTTCA
AAGAGCCTTGAAAGAAAACTTTGGCTACAACCAATCTGTTCACCAAATCAACCGTAGTGACTTTAGCAAACAA
GATTGGGAAGCACAAATTGACAAAGAATTATCTCAAAACCAACCAGTATACTACCAAGGTGTCGGTAAAGTAG
GCGGACATGCCTTTGTTATCGATGGTGCTGACGGACGTAACTTCTACCATGTTAACTGGTTGGTGGAGTCTGA
CGGCTTCTTCCGTCTTGACGCACTAAACCCTTCAGCTCTTGGTACTGGTGGCGGCGCAGGCGGCTTCAACGGT
TACCAAAGTGCTGTTGTAGGCATCAAACCTTAG

Figure 1. *Streptococcus pyogenes* pyrogenic exotoxin B (speB) Gene Sequence (Accession Number: L26148.1)

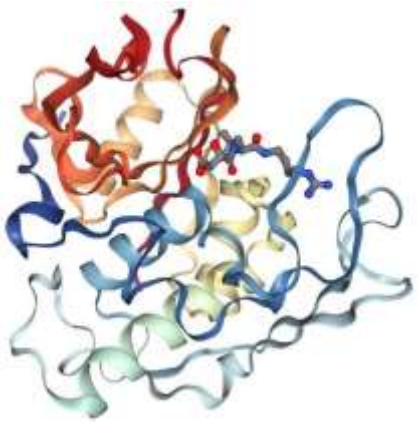

Figure 2. 3D structure of *Streptococcus pyogenes* speB protein visualized by PDB RCBS

The next step is primer design using primer3plus using FASTA derived from gene banks (Ethica et al., 2019). The primer design that emerged identified the possibility of hairpin and dimerformation that must be avoided (Ethica et al, 2019). The newly identified primers were used as input for web-based in silico PCR (http:// insilico.ehu.es/ PCR/) using all genomes of the Streptococcus genus taken from its data-based sources. A DNA band will appear, this band is the output of the program that displays the amplification results in silico. This step aims to clarify whether the in silico PCR product (amplicon) is specific to the S.pyogenes genome and is really part of the targeted gene.

RESULTS

The SpeB gene is a protein constituent that is a toxin that will cause damage and even death of the glomerulus due to the cysteine protease produced. Its highly virulent role causes the gene

sequence (Figure 1) to be used as the basis for primer design. The protein 3D structure of the SpeB protein is shown as a validity test if the genomic DNA sequence used to design the primers produces a folding (functional) protein (Figure 2) showing that the SpeB protein is folded and functional. This is a marker of bacterial proteins or for the development of vaccines or reagents using the protein as a target. The results of primer design using Primer3Plus obtained as a result of input SpeB gene sequence listed in Table 1.

Table 1. Primary design results using Primer3Plus

Primer pair No	Primer sequence	Tm (°C)	Amplicon Size (bp)	DNA of *Streptococcus pyogenes* strains Amplified (code number)	GC content	Dimer	Hairpin
1.	F5' TTGGGAAGCACAAATTGACA3'	60.1	157	68 - 80, 82, 84 - 86	40	-	-
	R3'GAAGCCGTCAGAGACTCCAC5'	60	157	68 - 80, 82, 84 - 86	60	-	-
2.	F5'TTGGGAAGCACAAATTGACA 3'	60.1	245	68 - 80, 82, 84 - 86	40	-	-
	R3'TGCCTACAACAGCACTTTGG 5'	59.9	245	68 - 80, 82, 84 - 86	50	-	-
3.	F5'TTGGGAAGCACAAATTGACA 3'	60.1	158	68 - 80, 82, 84 - 86	40	-	-
	R3'AGAAGCCGTCAGAGACTCCA5'	60.1	158	68 - 80, 82, 84 - 86	55	-	-
4.	F5'GTCGGTAAAGTAGGCGGACA3'	60.1	187	68-86	55	-	-
	R3'TGCCTACAACAGCACTTTGG 5'	59.9	187	68-86	50	-	-
5.	F5'GGTGCTGACGGACGTAACTT 3'	60.2	151	68-86	55	-	-
	R3'TGCCTACAACAGCACTTTGG 5'	59.9	151	68-86	50	-	-

There are 5 pairs of primers consisting of forward and reverse primers used for PCR amplification and can have a major influence on the sensitivity and specificity of the reaction. The use of Primer3Plus helps in obtaining data such as primer sequence, melting point, product size, GC content percentage and amplicon. The data in table 1 makes it easy to choose the right primer design with good quality. Based on the data presented, there are different primer sequences. Melting point temperature is between 59.9 - 60.2 ºC.

Qualified primer pairs were inputted in to in silico PCR amplification http:// insilico.ehu.es/ PCR/. In silico PCR is also called e-PCR which serves to provide an overview when the real primer

before the real PCR will be done, by doing this primer selection becomes more efficient. The 5th primer pair was then input into the in silico PCR to amplify the SpeB gene fragment from S.pyogenes.

Figure 3. Input primer design of the 5th primer pair in in silico PCR, amplifying SpeB gene fragment from *S. pyogenes*

Figure 4. The gene sequence results of the 5th primer pair were entered into in silico PCR and the following amplicon was obtained

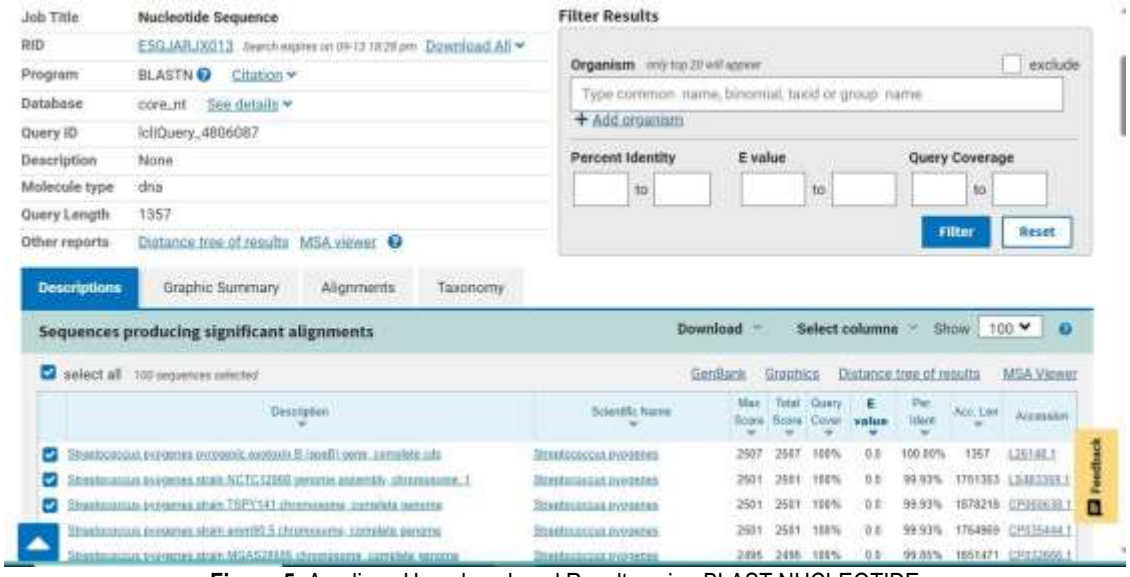

Figure 5. Amplicon Homology Level Results using BLAST NUCLEOTIDE BLAST: Basic Local Alignment Search Tool (nih.gov)

After performing in silico PCR, we checked the amplicon product that will appear on the electrophoresis gel as shown in Figure 4 with the amplicon in parallel position. The results of this study

are based on in silico studies or predictions using in silico PCR at http:// insilico.ehu.es/ PCR. The results of this in silico primer design can be used to support the success of DNA amplification using in vivo PCR as a way to early detect and identify the causative agent of opportunistic infections caused by bacteria. The results of blast using NCBI tools obtained 100% homology results, this indicates that SpeB is able to be a suitable primer for examination for the detection of Streptococcus pyogenes infection causing GNAPS.

DISCUSSION

The size of the primer sequence in general, the ideal primer has a length of between 18 to 30 oligonucleotides. This length is expected to be sufficient to remember the template at annealing temperature and get a specific sequence (Yustinadewi, 2018). If the primer is too short, it will reduce the specificity of the primer so that it easily sticks to the template with annealing temperatures that should not be, while if the primer is too long it does not affect the specificity significantly. The number of nucleotide bases produced in the primer is 19 - 20bp. This result is as required, so the possibility of primers sticking specifically is very large.

The size of the primary product obtained has a range of 151 - 245bp. According to (Riley et al, 2013) the product size is between 80 - 300 bases, so the primer product size meets the ideal requirements. However, primers with amplicon size close to 300 are the most stable primers. GC percentage is the percentage of guanine and cytosine in a primer provides information about the optimal temperature of the primer in the annealing process.

GC content should be in the range of 40 - 60% (Bustin et al, 2020). This will strengthen the attachment between the primer and the DNA template so it can be concluded that the more GC, the stronger it will be. Table 1 shows GC content between 45% - 55%, so 5 pairs of primers fall within the recommended range.

The order of complementary primers in primer design also needs to be considered because primer design should not contain intra-primer homology of more than 3 base pairs. This causes the reverse and forward primers to stick to each other, but it also allows for partial homology, where one primer sticks to the middle region of two primers that can interfere with hybridization or annealing (Ethica et al, 2019). All primer pairs were analyzed http://www.geneinfinity.org/sms/sms_primanalysis.html# to see the oligocalculator for the possibility of

hairpin and dimer. In all primers, hairpins and dimers did not occur, so all had the potential to become primers.

Table 2 shows all current Streptococcus strains stored in the databased in silico PCR program and serve as DNA templates for primer design with Primer3Plus.

Table 2 List of Streptococcus species genomes used as *in silico* PCR templates

Full list of *Streptococcus pyogenes* strains in Databased in silico PCR
68 *Streptococcus pyogenes* A20
69 *Streptoccocus pyogenes* Alab49 70
70 *Streptococcus pyogenes* HSC5
71 *Streptococcus pyogenes* M1 476 DNA
72 *Streptoccocus pyogenes* M1 GAS
73 *Streptococcus pyogenes* MGAS10270
74 *Streptococcus pyogenes* MGAS10394
75 *Streptoccocus pyogenes* MGAS10750
76 *Streptoccocus pyogenes* MGAS15252
77 *Streptococcus pyogenes* MGAS1882
78 *Streptococcus pyogenes* MGAS2096
79 *Streptococcus pyogenes* MGAS315
80 *Streptococcus pyogenes* MGAS5005
81 *Streptococcus pyogenes* MGAS6180
82 *Streptococcus pyogenes* MGAS9429
83 *Streptococcus pyogenes* NZ131
84 *Streptococcus pyogenes* SSI-1
85 *Streptococcus pyogenes* str. Manfredo
86 *Streptococcus pyogenes* strain MGAS8232

The number of amplicon bands that appear from the results of in silico PCR input is 19 pieces and is specific to Streptococcus pyogenes only. From the number of amplicons and amplicon positions, the amplicon locations are aligned. This aligned location helps in the selection of the desired primer pair and minimizes non-target products. This proves that these primers are highly specific, sensitive and efficient when used as primers. The SpeB gene is highly conserved by looking at the 100% homology in the species group (Figure 5). After performing in silico PCR, we checked the amplicon product that will appear on the electrophoresis gel as shown in Figure 4 with the amplicon in parallel position. The results of this study are based on in silico studies or predictions using in silico PCR at

http:// insilico.ehu.es/ PCR. The results of this in silico primer design can be used to support the success of DNA amplification using in vivo PCR as a way to early detect and identify the causative agent of opportunistic infections caused by bacteria.

CONCLUSION

Primer design obtained in silico produced the best primers for SpeB gene detection in Streptococcus pyogenes bacteria. These primers include forward primer F5' GGTGCTGACGGACGTAACTT 3' and reverse primer R3' TGCCTACAACAGCACTTTGG 5'.The primer design was able to amplify the SpeB gene region with an amplicon size of 151. These primers can be used in the examination of GNAPS by PCR method so that it is earlier to be treated so that it will reduce the mortality rate due to complications of GNAPS.

Conflict of Interest

The authors declare that they have no conflict of interest.

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