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speB GENE AS A SPECIFIC GENETIC MARKER FOR EARLY DETECTION OF RHEUMATIC HEART DISEASE IN HUMAN

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Abstract

Streptococcus pyrogenic exotoxin B gene *(speB)* is chromosomally encoded pyrogenic and cardiotoxic virulence factor of *S. pyogenes*. Exotoxin B is produced only in a secreted form, as a 40 KD proprotein, which is subsequently processed to 28 KD in the mature form. *Streptococcus pyogenes* infection in human, causes initially pharyngitis due to inhalation of aerosols emitted by infected persons, develops rheumatic fever which leads to the rheumatic heart disease (damage of heart valves). The available detection methods are bacterial culture, β-hemolysis, bacitracin sensitivity, hippurate test, phadebact test, CRP (C-reactive protein), ESR and PCR. All these methods are either expensive or non-confirmatory and have some limitations. Available PCR methods take more time and require other test to confirm the disease. Our PCR based detection of *Streptococcus pyogenes* in human using specific primers of *speB* gene completes overall analysis in 80 min which is the minimum time reported so far for the confirmation of the disease. Amplicon of 423bp of *speB* gene can be used as a specific genetic marker as it does not show homology with other organisms for early detection of rheumatic heart disease. Our method is specific virulence gene based which is quick, economical and more sensitive as compared with other methods.

Key words: RHD marker, Rheumatic heart disease, *speB* gene, *S. pyogenes*.

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INTRODUCTION

Rheumatic heart disease (RHD) is a complication of rheumatic fever in which the heart valves (mitral and aortic) are damaged. Rheumatic fever is an inflammatory disease that begins with strep throat. The main causative organism for this disease is *Streptococcus pyogenes*. *Streptococcus pyogenes* affects many of the body's connective tissues especially of the heart, joints, brain or skin. The most common symptoms of rheumatic infection are joint pain, fever, chest pain or palpitations caused by heart inflammation (carditis), jerky uncontrollable movements (Sydenham's chorea), a rash and small bumps (nodules) under the skin (6, 8). Increasing resistance of *S. pyogenes* to antibiotics has called for the search of new preventive methods and therapies (23). The adherence of bacteria to the host cells as the initial event in the pathogenic process is a potential target for anti-adherence therapy, in which analogues of receptor molecule are used to prevent the bacteria from binding to the host cells (9, 10). The patient remains infected for weeks even after symptomatic resolution of pharyngitis and may serve as a reservoir of infection to others (20, 21, 22). Therefore, accurate detection and diagnosis of streptococcal infection is essential for prevention, therapeutic and clinical management of the disease (11, 12). Most of the current tests rely on several laboratory methods in addition to clinical symptoms, history and geographic location of the patient which are not much useful for therapy and prognosis of the disease (2, 3, 5). Recent developments in new diagnostic tools, however, have opened new avenues for vast improvements in the diagnosis of rheumatic heart infection (3).

The epidemiology of acute rheumatic fever (ARF) is linked with group A beta-haemolytic streptococcus pharyngitis and both have a maximum incidence in the age group of 5-15 yrs (4, 5). In developed countries, RHD has become an uncommon health problem during the past two decades. In contrast, in the countries like India, the middle-east and sub Saharan Africa, RHD remains a leading cause of heart problems in both children and adults (4, 6). According to the world health organization (WHO), at least 15.6 millions people are affected with RHD in the world. Out of the 5 lakh individuals of ARF, 3 lakh develop RHD and 2.3 lakh deaths are attributed to RHD/ARF. Recent data from India suggest that a large number of cases of RHD are still seen frequently in young children under the age of 10 years (1, 13, 14).

Molecular diagnostics are revolutionizing the clinical practice of infectious disease. The introduction of polymerase chain reaction (PCR) based DNA amplification in the early nineties is being used for the diagnosis of infectious diseases (17, 18, 19). Compared to serology, PCR-based diagnosis is more specific and sensitive which allows early detection of rheumatic heart disease (7, 8,16). In addition to various virulence factors, *S. pyogenes* also produces several super-antigens like erythrogenic toxins. The erythrogenic toxins secreted by *S. pyogenes* are antigenically distinct extracellular toxins known as streptococcal pyrogenic exotoxins (*spe*), which include A, B C, F, G, H and J type (9). These toxins are believed to be associated with pyrogenicity. Furthermore, these toxins have extra ability to cause myocardial necrosis. Identification of *speB* gene serves as a marker for serotyping. *speB* (streptococcal pyrogenic exotoxin B) is chromosomally encoded pyrogenic and cardiotoxic virulence factor of *S. pyogenes* (15). It degrades host extra-cellular matrix surface pro-

teins (10). SpeB has been considered to be produced only in a secreted form, as a 40 KD proprotein, which is subsequently processed to the 28 KD in the mature form (11, 12). The *speB* gene is chromosomally located on every group A streptococcus strain and consists of a 1196 bp open reading frame yielding 371 amino acid polypeptide with a predicted molecular weight of 40 KD. SpeB is secreted strictly in the late log or early stationary phase of growth as a proteinase precursor which is proteolytically cleaved to mature active form. SpeB is also found on the surface of the bacteria and possess glycoprotein and laminin binding activities (13).

MATERIALS AND METHODS

Sample collection and chemicals

The bacterial strain M 140 was taken from IMTECH Chandigarh. The patient's throat swab samples were collected from Sufdurjung Hospital and experiments were performed at NCDC. PCR chemicals and Taq polymerase were obtained from Bangalore GeNei, India. Tris and EDTA were purchased from Sigma-Aldrich, USA. Other chemicals were analytical reagent grade. DNA purification kit (including GFX column) was purchased from Amersham Biosciences, UK Ltd. Primers were synthesized from TCGA (The Centre for Genomic Application), India.

Sample preparation

The suspected patient throat swab samples were dissolved in 500µl of autoclaved TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and heated at 94 ^{O}C for 3 min. After heating, all tubes were centrifuged at 4000xg for 2min and pellet was washed with autoclaved water (centrifugation) and finally dissolved in 50 µl of autoclaved water. 3 µl of this solution (bacterial DNA) was added in 22 µl of PCR reaction mixture (pellet may also be dissolved directly into 25 µl PCR reaction mixture if concentration of bacterial DNA is less) and transferred into PCR tubes for PCR. For quantification and purity ($A_{260/280}$) determination, genomic DNA sample was dissolved in water (instead of PCR reaction mixture) and measured by Nanodrop spectrophotometer. The genomic DNA was also isolated from 18 h cultured *S. pyogenes* (strain M140, IMTECH Chandigarh) in brain heart infusion broth at NCDC using phenol chloroform method for standardization of PCR method (24).

Amplification of target gene

The sequence of *speB* gene of *Streptococcus pyogenes* was retrieved from NCBI and checked for the homology with other organisms. The *speB* gene based forward primer (5'-GTA GCAACACATCCTGTAGCTGCA-3') and reverse primer (5'- AGGTGCAC GAAGCG CAG AAG ATAT -3') were synthesized and the PCR was carried out with the following steps: Initial heating at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 7s, annealing at 55°C for 10s, extension at 72°C for 5s and final extension for 3 min after the last cycle. The PCR was carried out in 25µl reaction mixture containing 1 x assay buffer, 1mM of dNTP (0.25mM of each dATP, dGTP, dCTP and dTTP), 0.2 µM each of forward and reverse primers, genomic DNA (100ng) or heated bacterial cells, 0.75 units Taq polymerase and Milli Q water. Sharp band was visualized in UV light corresponding to 423 bp in DNA marker. The amplified PCR product was purified using GFX column and agarose gel electrophoresis of PCR product was carried out in 1.5% agarose gel at 80V. The purified PCR product was sequenced by TCGA (THE CENTRE FOR GENOMIC APPLICATION) and homology of the *speB* gene sequence of *S. pyogenes* was confirmed by BLAST.

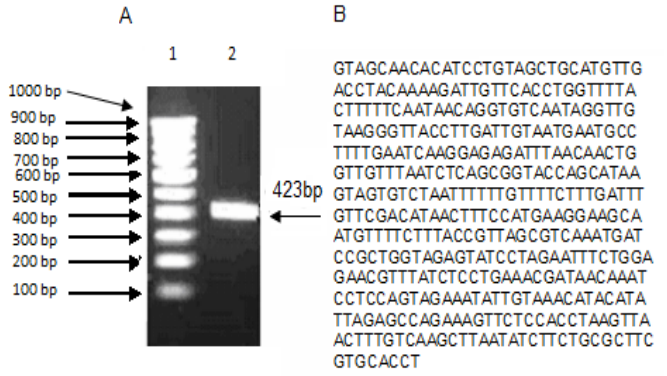

Figure 1. [A] Agarose gel electrophoresis (1.5%) of PCR product. Lane1: DNA marker 100bp; Lane2: GFX column purified PCR product (423bp). [B] Gene sequence of PCR product (423bp) using reverse primer.

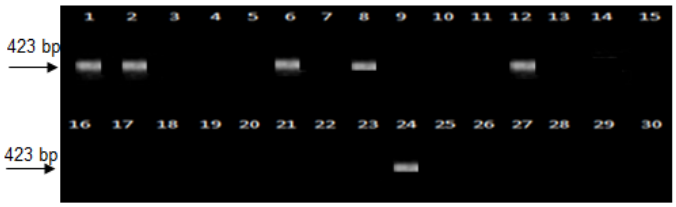

Figure 2. Agarose gel electrophoresis (1.5%) of suspected RHD patients. Lane 1 and lane 2 (control) and lane 6, 8, 12 and 24 show single band corresponding to 423bp of DNA marker (infected patients) whereas lane 3-5, 7, 9-11, 13-23 and 25-30 showed no bands (normal person).

RESULTS AND DISCUSSION

The DNA sequence of 423bp amplicon after BLAST shows similarity with *speB* gene of *S. pyogenes* which confirmed that the right fragment within *speB* gene had been amplified correctly. Primers used in this experiment were specific and amplify only the specific region under specific conditions (Fig.1). Several diagnostic methods for the diagnosis of *S. pyogenes* in patient samples like culture, CRP (C-reactive protein), ESR (Erythrocyte sedimentation rate), agglutination test, antibiotic sensitivity etc. are available but all these tests are time consuming and have some limitations. The results suggest that *speB* gene can be used as specific genetic marker for the diagnosis of *S. pyogenes*. Amplicon of 423 bp of *speB* gene does not show homology on BLAST with other organisms and therefore it can be used as a genetic marker for early detection of *S. pyogenes* causing rheumatic heart disease. The diagnosis of the suspected strep throat patient (28 samples and the 2 controls) was carried out with different methods (Table 1) which are currently being used in labs as well as with our PCR based method using *speB* as genetic marker (Fig.2). Sample 1 and 2 used as control (positive test) using genomic DNA isolated from *S. pyogenes* culture. Sample 6, 8, 12 and 24 showed positive by our PCR method and their bands correspond to 423bp of *speB* gene of *S. pyogenes*. But sample 8 and 30 showed catalase positive test where as others showed negative which sug-

Table 1. Diagnosis of suspected patients by different available methods and PCR using *speB* gene as a specific genetic marker.

Sample No.	Present available methods (16)				PCR with marker	Normal/ Infected patient
	Microscopic examination (Gram +)	Culture on blood agar β-hemolysis	Enzyme test Catalase	Antibiotic susceptibility Bacitracin	*speB gene* (423 bp)	
1 Control	+	+	-	+	+	C
2 Control	+	+	-	+	+	C
3	-	-	-	-	-	N
4	-	-	-	-	-	N
5	-	-	-	-	-	N
6	+	+	-	+	+	P
7	-	-	-	-	-	N
8	+	+	+	+	+	P
9	-	-	-	-	-	N
10	-	-	-	-	-	N
11	-	-	-	-	-	N
12	+	-	-	+	+	P
13	-	-	-	-	-	N
14	-	-	-	-	-	N
15	-	-	-	-	-	N
16	+	-	-	-	-	N
17	-	-	-	-	-	N
18	-	-	-	-	-	N
19	-	-	-	-	-	N
20	-	-	-	-	-	N
21	-	-	-	-	-	N
22	-	-	-	-	-	N
23	-	-	-	-	-	N
24	+	+	-	+	+	P
25	-	-	-	-	-	N
26	-	-	-	-	-	N
27	-	-	-	-	-	N
28	+	-	-	-	-	N
29	-	-	-	+	-	N
30	-	-	+	-	-	N

Control = C, Normal individuals = N, Infected patients with *S.pyogenes* = P.

gest that catalase is not specific test for *S. pyogenes* (16, 21, 24, 25). Other available tests also have some limitations [β-hemolysis (sample 12), Gram staining (sample 16 and 28) and Bacitracin (sample 29)] and give false results whereas our method uses *speB* gene based specific primers which give amplicon of 423bp. Hence, *speB* gene can be used as marker for early diagnosis of rheumatic heart disease to prevent damage of mitral and aortic heart valve. Sample 12 was collected from the patient who already had rheumatic fever. The present method is simple, economical and fast as it takes only 80 min including electrophoresis for confirmation of the disease.

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Other articles in this theme issue include references (26-53).

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