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Genotyping of *Streptococcus Pyogenes* Isolates using Optimized RAPD-PCR Protocol

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Abstract

Introduction: *Streptococcus pyogenes* causes a variety of infectious and non-infectious diseases. Typing of *S. pyogenes* isolates is one of the essential tools in the epidemiological studies of this bacterium. Random Amplified Polymorphic DNA (RAPD) is a rapid, easy and inexpensive PCR-based typing technique. Low reproducibility of RAPD-PCR is the main disadvantage of this method which will be resolved by optimization of RAPD-PCR protocol.

Materials and methods: In this study, optimization of RAPD-PCR protocol including DNA extraction method, primer type, concentrations of PCR reagents, and PCR program was performed using the factorial design of experiments for *S. pyogenes* ATCC 19615 as a standard strain. Then, sixteen *S. pyogenes* isolates were genotyped by using optimized protocol. Typability, reproducibility, and discriminatory power of the optimized protocol were examined.

Results: Among three DNA extraction methods and seven primers that were used, modified set buffer DNA extraction method and P14 primer were selected, respectively. Optimum concentration of PCR reagents were 3 mM $MgCl_2$, 150 pmol primers, 0.2 mM dNTPs, 10 ng template DNA, and 2 U Taq DNA polymerase and the optimum PCR program consisted of an initial denaturation for 4 min at 94°C followed by 45 cycles of 1 min at 94°C, 2 min at 31°C, 2 min at 72°C, and a final extension at 72°C for 10 min. Results of optimized RAPD-PCR were reproducible for *S. pyogenes* ATCC 19615 and all *S. pyogenes* isolates. Calculated discriminatory power was satisfactory (DI=1). Sixteen *S. pyogenes* isolates belonged to sixteen strains which were classified into 3 main clusters on a similarity level of 14%.

Discussion and conclusion: A suitable and reproducible RAPD-PCR protocol was obtained for genotyping of *S. pyogenes* isolates using RAPD-PCR optimization. The optimized protocol in the present study can be used in subsequent experiments on RAPD-PCR profiling for epidemiological study of *S. pyogenes* isolates.

Key words: Epidemiology, Factorial Design, Genotyping, RAPD-PCR, *Streptococcus Pyogenes*

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Introduction

Streptococcus pyogenes causes various infectious and non-infectious diseases such as cellulitis, erysipelas, necrotizing fasciitis, puerperal fever, sepsis/bacteremia, streptococcal pharyngitis, streptococcal pyoderma, toxic shock syndrome, and scarlet fever. Rheumatic fever and acute glomerulonephritis can develop as complications of inadequately treated streptococcal pharyngitis.

Among the most severe life-threatening diseases are those caused by *S. pyogenes*. While the rate of many diseases has decreased in developed countries, developing countries suffer a high incidence of *S. pyogenes* diseases with millions of deaths yearly (1).

Epidemiological studies examine the distribution of diseases and pathogens in societies and identify the origin of diseases, and provide solutions for disease prevention and control. Typing of *S. pyogenes* isolates is one of the essential tools in the epidemiological studies of this bacterium.

Typing systems are evaluated based on typability, reproducibility, and discriminatory power. A good typing method should differentiate among unrelated strains and generate reproducible and unambiguous results that can be interpreted easily. Furthermore, it should be inexpensive, easy and applicable to a broad range of microorganisms.

Several methods have been developed for *S. pyogenes* typing such as M protein gene (*emm*) typing (2, 3, 4), restriction fragment length polymorphism (RFLP) (3), pulsed-field gel electrophoresis (PFGE) (2, 5), multi-locus sequence typing (MLST) (4, 5), and multiple loci VNTR analysis (MLVA) (5).

One of the gold standard methods to characterize *S. pyogenes* isolates is *emm* typing that is based on sequencing of N-terminal hyper variable region of the *emm* (6), and the sequencing requires an equipment that is not available in most laboratories.

RAPD-PCR is a simple, rapid, easy, and inexpensive method that can be performed in a moderate laboratory (7). In this method, a single short primer (8-12 nucleotides) is used in each reaction which its melting temperature (T_m) is low (8). Primers can attach randomly to several DNA sequences in the genome (9). The number and the positions of binding primer sites are unique for each bacterial strain (8). Amplified segments of DNA in RAPD PCR technique are random. Differences between the generated RAPD patterns from the different DNAs indicate polymorphism between strains. Prior knowledge of the genome under research is not necessary (10). Therefore, this technique is suitable for molecular typing of unknown strains. The main disadvantage of RAPD-PCR method is low reproducibility of the results (11). Low intra-laboratory reproducibility is because of very low annealing temperature in RAPD-PCR reaction and low inter-laboratory reproducibility is as a result of the sensitivity of RAPD-PCR reaction to very little differences in reagents, protocols, and equipment (8).

Most of the variation that is sometimes observed in RAPD-PCR pattern will be eliminated by the optimization of the RAPD reaction and PCR protocol (12-14).

In the present study, the optimization of RAPD protocol was performed to obtain reproducible RAPD patterns for fingerprinting of *S. pyogenes* isolates.

Materials and Methods

Bacterial Strains and Culture: We studied 16 well-characterized *S. pyogenes* isolates which were collected from the throat of children aging from 5 to 15 years in 2011 (15). Reactions with *S. pyogenes*

(ATCC 19615) and without DNA were also used as positive control and negative control, respectively. Bacteria from frozen stocks were cultured in Tryptic Soy Broth (TSB) (Merck, Germany) and incubated overnight at 37°C in a candle jar.

DNA Extraction Optimization: DNA was extracted by three methods of boiling (16), modified freeze-thaw (17), and modified set buffer (18). Concentration and purity of extracted DNA were measured by NanoDrop (Nanodrop2000, Thermo Scientific) and its quality was determined by 0.8% agarose gel containing 0.1 µl/ml of DNA Green Viewer (*Pars Tous Zist Fanavar, Iran*). Extracted DNA by these three methods was used as template in RAPD-PCR reaction and results were compared to each other.

RAPD-PCR Optimization: Optimization of RAPD-PCR protocol was performed for *S. pyogenes* ATCC 19615. Then, sixteen *S. pyogenes* isolates were genotyped by using optimized protocol.

Primer Selection: Seven primers (FazaBiotech, Tehran, Iran) were screened for RAPD typing of *S. pyogenes* ATCC 19615 and *S. pyogenes* isolates (Table 1). The optimum annealing temperature for each primer was determined in an automated gradient thermal cycler (PeQSTAR 96 Gradient, Peqlab) by using the same PCR program and reagent concentrations. The 25 µl reaction mixture contained 20 mM Tris-HCl, pH 8.8, 5 mM KCl, 3 mM $MgCl_2$, 10 pmol primers, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase (CinnaGen, Tehran, Iran) and 20 ng template DNA.

The PCR program consisted of an initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 35±5°C for 1 min, extension at 72°C for 2 mins and a final extension at 72°C for 10 mins. The best primer was selected in this step and other factors were optimized for that one.

Table 1- Used Primers for the Optimization of RAPD-PCR Protocol of *S. Pyogenes*

Name of primer	Sequence 5´⟶3´	Size (bp)	GC content (%)	T_m [a] (°C)	Range No. of distinct RAPD product	reference
KIT	CCCGTCAGCA	10	70	34	4	(19)
P17	GATCTGACAC	10	50	30	3	(20, 21)
P14	GATCAAGTCC	10	50	30	8	(20-22)
OPA5	AGGGGTCTTG	10	60	32	5	(23)
OPA13	CAGCACCCAC	10	70	34	8	(24)
OPA14	GACCGCTTGT	10	60	32	1	(24)
H2	CCTCCCGCCACC	12	83.3	44	7	(25)

[a] The theoretical melting temperature was calculated from the formula: $T_m = (4 \cdot (G + C)) + (2 \cdot (A + T))$.

Optimization of RAPD-PCR Reagent and Cycling Program: Factorial design of experiments was used for determining the effect of different concentrations of $MgCl_2$, Taq DNA polymerase, dNTP and primer on RAPD-PCR profile (Table 2). The significance of the effect of each factor was determined using ANOVA (SPSS 24.0 for Windows; SPSS, Chicago, IL, USA).

Assessment of the effect of template DNA concentration on RAPD-PCR profile was performed using ten different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 ng).

Optimization of annealing and extension times were carried out using factorial design and four levels were evaluated for each factor (1, 1.5, 2, 2.5 mins). To determine the optimum number of cycles, four sets of experiments (30, 40, 45, 50 cycles) were examined.

Table 2- Factorial Design of Experiments for the Optimization of the RAPD-PCR Reagent Concentrations

factor	concentration	No. of experiment	No. of distinct RAPD bands (mean±SD)	Sig
$MgCl_2$	1.5 mM	24	0±0.0	0.000
	2.5 mM	24	6.50±3.72	
	3 mM	24	9.33±2.06	
dNTP	200 µM	36	6.56±5.12	0.04
	400 µM	36	4.00±3.96	
Taq DNA polymerase	1.5 U	24	4.00±3.84	0.04
	2 U	24	5.17±5.03	
	2.5 U	24	6.67±5.35	
primer	1.5 µM	18	3.83±3.76	0.025
	3 µM	18	5.67±3.55	
	4.5 µM	18	7.83±2.31	
	6 µM	18	9.33±2.06	

Effect of DMSO and Thermal Cycle Device on RAPD Profile: For determining the effect of dimethyl sulfoxide (DMSO (Merck, Germany)) on RAPD profile, three concentrations (6%, 8%, and 10% V/V) of DMSO were used in RAPD-PCR reactions. For investigating possible effect of thermal cycle device on RAPD profile, two different thermal cycler devices (PeQSTAR 96 Gradient, Peqlab and Primus 25 advanced, Peqlab) were used.

Analysis of RAPD-PCR Products: The amplified products of RAPD-PCR along with the DNA molecular weight marker (100bp Plus DNA Ladder; SinaClon) were separated on 1.5% agarose gel containing 0.1 µl/ml of DNA Green Viewer. Analysis of PCR products on agarose gel was performed using GelCompar II software. The dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) method based on the Pearson correlation coefficient.

Discriminatory power of optimized RAPD protocol was calculated using Simpson's diversity index.

Reproducibility of RAPD profiles was obtained by comparing the fingerprints generated from three replicates of RAPD-PCR reaction for *S. pyogenes* ATCC 19615 and sixteen *S. pyogenes* isolates. Experiments were repeated 3 times at 4 weeks intervals.

Results

Average concentrations of extracted DNA by boiling, modified freeze-thaw and modified set buffer methods were 900, 400, and 120 (ng/µl) and their average purities (A260/A280) were 1.5, 1.6, and 1.9, respectively. We obtained the best quality of extracted DNA with the modified set buffer method, and extracted DNA by this method provided the highest number and intensity of bands in RAPD-PCR reaction. Thus, this method was found to be the optimum DNA extraction technique for RAPD analysis.

The optimum annealing temperature of primers P14, P17, OPA13, OPA14, H2, OPA5, and KIT were obtained 31°C, 31°C, 35°C, 31°C, 34°C, 33°C and 31°C, respectively. Among these seven primers, H2, OPA13, and P14 lead to the best result and based on the number, intensity and size range of RAPD bands and less smear formation, P14 primer was selected as the best one. So, the amplification conditions were optimized using primer P14.

The optimum concentration of PCR components that generated the highest number and intensity of bands were 3 mM $MgCl_2$, 150 pmol primer P14, 0.2 mM dNTPs, 10 ng template DNA, and 2 U Taq DNA polymerase. RAPD-PCR reaction was inhibited in a high concentration of DNA (40 ng<), and intensity of bands decreased in a low concentration of DNA (<10 ng).

The yield of RAPD-PCR products decreased in low concentrations of primer, $MgCl_2$, and Taq DNA polymerase and non-specific products and smearing were formed in an excess concentration of Taq DNA

polymerase (2.5 U≤). Band number decreased by increasing dNTP concentration up to 0.4 mM.

ANOVA showed RAPD profiles were significantly affected by the concentrations of $MgCl_2$ (Sig=0.000, P<0.0001), primer (Sig=0.025, P<0.05), dNTP, and Taq DNA polymerase (Sig=0.04, P<0.05). Based on the results of this study, $MgCl_2$ had the most effect on the RAPD profile.

The optimum cycling program consisted an initial denaturation at 94°C for 4 mins followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 31°C for 1 min, extension at 72°C for 2 mins and a final extension at 72°C for 10 mins.

Number and intensity of bands were improved by increasing annealing and extension time up to 2 mins, respectively. Annealing time longer than 2 mins caused non-specific products formation. RAPD profiles using 2 mins extension time were identical to RAPD profiles using extension time longer than 2 mins.

The intensity of bands was also improved by increasing the number of cycles up to 45 cycles. The lower intensity of bands and non-specific products were observed in 50 cycles.

DMSO increased the number and intensity of RAPD bands. The best DMSO concentration was 10%. Our results show that thermal cycler device type had no effect on the RAPD profile.

All of *S. pyogenes* isolates were typable using primer P14. A total of 16 different RAPD patterns were found among the 16 *S. pyogenes* isolates studied in this work. RAPD profiles of sixteen *S. pyogenes* isolates and *S. pyogenes* ATCC 19615 using primer P14 are shown in Figure 1. The patterns of amplification demonstrate DNA polymorphism among all isolates of *S. pyogenes* used in this study. Based on constructed dendrogram (Figure 2), sixteen *S. pyogenes* isolates belonged to sixteen strains which were classified into 3 main clusters on a similarity level of 14%. Calculated discriminatory power for optimized RAPD protocol was very satisfactory (DI=1). The identical fingerprints generated from three replicates of RAPD-PCR reaction for *S. pyogenes* ATCC 19615 and sixteen *S. pyogenes* isolates using optimized protocol indicated high reproducibility of RAPD-PCR results.

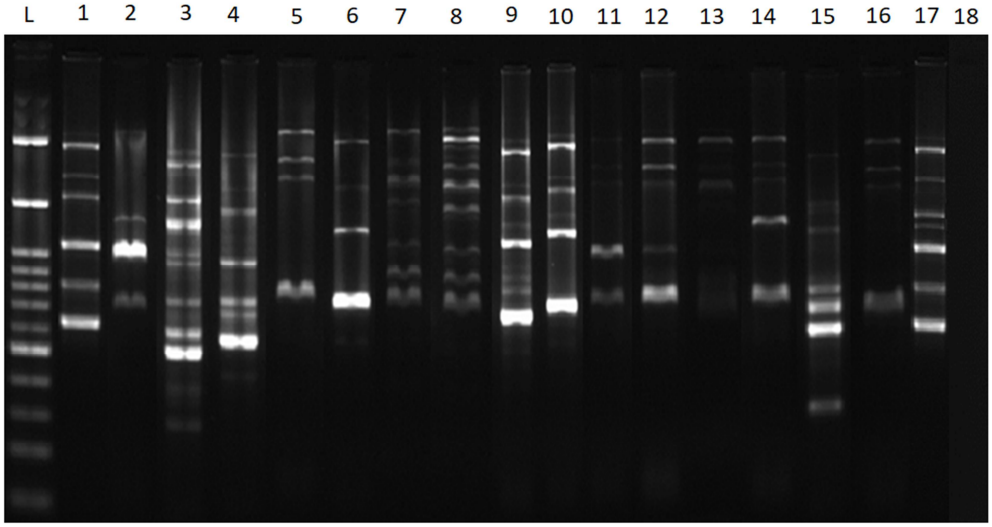

Fig. 1- Results of RAPD fingerprinting of *S. pyogenes* isolates and *S. pyogenes* ATCC 19615 using optimized protocol by P14 primer. DNA molecular weight marker (L) (1 kb DNA ladder; Fermentas) and RAPD profile of *S. pyogenes* clinical isolates (1-16), standard strain of *S. pyogenes* (17) and negative control (18) are shown from left to right.

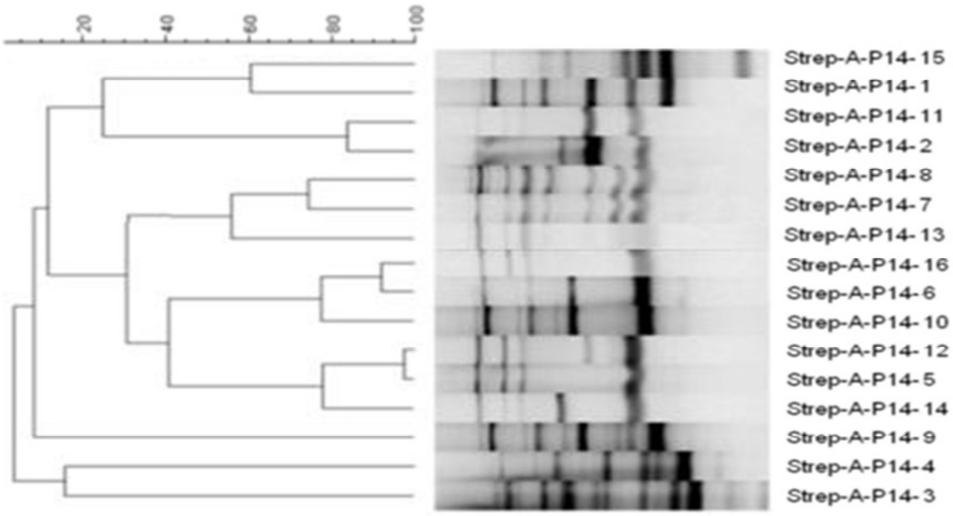

Fig. 2- Constructed dendrogram using the UPGMA method and GelCompar II software based on RAPD bands of sixteen *S. pyogenes* isolates.

Discussion and Conclusion

Reproducibility of RAPD-PCR method can be improved by the optimization of the protocol (12-14). In our study, optimization of RAPD-PCR protocol was performed using the factorial design of experiments and reproducible RAPD-PCR protocol was obtained for genotyping of *S. pyogenes* isolates.

We found that the quality and purity of the template DNA have a great effect on the generation and resolution of amplified products in RAPD-PCR reaction. Our findings were in accordance with the results of some other studies (26, 27). It is seemed impurities with extracted DNA act as inhibitors during RAPD-PCR.

DNA concentration is a critical factor in RAPD-PCR reaction which can influence the number and intensity of products resulting in different fingerprints. Jain et al. (2010) also showed excess DNA concentration result in inhibition of RAPD-PCR reaction (28). Perry et al. (2003) reported that excess template can result in suppression of the amplification process due to competition between template DNA and first-round amplicons and a relative shortage of primers (29).

Among seven primers which were examined, P14 primer produced the most discriminative RAPD patterns which were consistent with previous research results (22). The lower and higher annealing temperature of optimum annealing temperature for P14 primer resulted in the formation of non-specific products and a significant reduction in band number, respectively.

In the present study, $MgCl_2$ had the most effect on the RAPD profile. $MgCl_2$ concentrations lower than 3 mM reduced the yield of RAPD-PCR products which was in agreement with the previous research findings (30, 31). Results of factorial design experiments showed that there is a direct correlation between $MgCl_2$ and primer, Taq DNA polymerase, and dNTP concentration in RAPD-PCR reaction. In order to obtain the best results, $MgCl_2$ concentration should be increased by increasing each of these reagents (primer, Taq DNA polymerase, and dNTP). $MgCl_2$ is the co-factor of the Taq DNA polymerase enzyme and interferes in some functions such as binding primer to template DNA, denaturation of template DNA and accuracy of Taq DNA

polymerase activity (31). Since dNTPs sequester Mg^{2+} ions, by increasing dNTP concentration in a reaction, would require an enhancement in the concentration of $MgCl_2$. $MgCl_2$ stabilizes primer annealing; therefore, the concentration of $MgCl_2$ has a large effect on the specificity and yield of a reaction (31). As Saiki reported, fidelity of Taq DNA polymerase reduces in excess $MgCl_2$ concentration that can result in non-specific products formation (31).

The yield of RAPD-PCR products and the intensity of bands were decreased in a very low concentration of primer as a result of lack of sufficient primer for amplification of products. These results were in agreement with the findings of some other studies (32, 33). Excess concentration of the primer leads to the primer-dimer formation (33, 34) and mismatches enhancement between the primer and the template DNA that results in increasing non-specific products formation.

The excess Taq DNA polymerase results in non-specific amplifications and smear formation while lower quantities than the necessary ones lead to the amplification failure or deficient amplification. Skorić et al. (2012) reported increasing the Taq DNA polymerase concentration provided an enhancement in the number and intensity of the detectable bands (14).

Band number decreased by increasing dNTP concentration. Magnesium ion is required as a co-factor for Taq DNA polymerase. dNTPs can reduce the amount of free magnesium ions present in a PCR reaction (35), thus, higher quantities than the necessary ones lead to inhibition of RAPD-PCR reaction as a result of reduced enzyme activity.

There is an interaction between the annealing time and the GC content of the primer. For primers containing high GC content, the amount of RAPD-PCR products is increased considerably by increasing annealing time (12). GC content of the primer P14 is 50%. Therefore, this primer required relatively long annealing time. The number of bands increased in longer annealing time for primer P14 as a result of increased primer binding to template DNA, but, non-specific products were observed in annealing time of 2.5 min.

There is a direct correspondence between the extension time and the maximum size of a fragment that is amplified. Longer extension time is required for the amplification of long PCR products (12). RAPD-PCR products are fragments with different length. By increasing the extension time up to 2 min in case of primer P14, the intensity of long products was improved as a result of complete amplification of long RAPD-PCR products.

Band intensity was improved as a result of more amplification of products by increasing number of RAPD-PCR reaction cycles up to 45. Low intensity of bands in 50 cycles may be attributable to Taq DNA polymerase inactivation over time or be an indicative that some other components in the reaction mixture become limiting at high cycle numbers. The accuracy of Taq DNA polymerase was reduced in very high cycle number, so non-specific products formation occurs that is in agreement with Fraga Nodarse *et al*. (2004) (36).

DMSO reduces the secondary structure of DNA, enabling strand separation, which can affect Taq DNA polymerase activity (37) and increases the number and intensity of bands.

Results of our study showed that RAPD-PCR protocol optimization could resolve the low reproducibility problem of this method. The optimized protocol in the present study can be used in subsequent experiments on RAPD-PCR profiling for epidemiological study of *S. pyogenes* isolates. Polymorphism in *S. pyogenes* isolates can be generated by mutation.

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