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Single-base difference at 3' end in a primer pair and its effect on diagnostic result on human genome with reference to Schizophrenia

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ABSTRACT:

Objective and Method – To investigate the effect of single - base difference (C- G) at the 3′–end of a primer on the diagnosis of a particular disease, if any. Our study involving a pair of the primers which represented a plieotropic gene- a transcription repressor gene on chromosome 1 that codes for a component of MeCP1 protein complex. The primer taken as standard, was an amplification of a 126 (base pair) region of a human gene P66beta. Due to trinucleotide (CAG) expansion, this gene is reported to be associated with disease schizophrenia in many population studies and thus PCR amplification of DNA of this gene demands precision for identification. **Results** – The primers with the C-G difference gave absolutely different PCR amplification - product results at all the different annealing temperatures (50-58^{o}C) tested than expected. The primers having single C-G difference at the 3'- end produced specific polymerase chain reaction (PCR) fragment at some of the annealing temperatures used, absolutely in contradiction with the expected ones identified as standard to diagnose the disease. **Conclusion** – We conclude that human genome possess many plieotropic genes corresponding to multiple phenotypic expressions in which even a single -base difference can create an absolute diagnostic error in identification. This makes the exact and proper primer sequence essential especially of those primers which are to be used in sensitive diagnostic techniques for identifying of molecular diseases as schizophrenia.

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I.INTRODUCTION

Of all the latest molecular techniques used to identify and study genes and their function at DNA level ,the most reliable technique of the times from precision point of view is PCR- A sensitive and powerful method known as DNA Polymerase Chain Reaction (PCR) can amplify specific sequences of template DNA by more than a million fold [Saiki RK; 1988]. Based on the principal of replication, 20–30 cycles of DNA synthesis are programmed in PCR machine, each cycle requires three stages: first, denaturation of DNA at high temperature (90–96ºC), second- annealing of oligonucleotide primers at a lower temperature (usually at 40 to 60ºC), and finally, extension of primers at 72˚C by *taq* DNA polymerase, a heat stable enzyme. The precision of the primers play an important role in the perfect amplification of PCR product needed to be identified in genomic DNA. Even a single –base substitution in similar oligonucleotides resulting in difference at the 3'–end may or may not be extended and serve as primers in the Polymerase Chain Reaction (PCR) due to lack of intrinsic 3' to 5' exonuclease activity of most of the DNA polymerases used in PCR (Newton CR; 1994). Factors including the nature of the difference, the kinetics of association and dissociation of primer-template DNA duplex at the annealing and extension temperatures and the effect of a substituted base on the stability of the duplex DNA formed effect the efficiency of such primers. Several investigators have begun to evaluate the possible effects of substitutions in their primers (Gibbs RA; 1989, Eiken HG; 1991, Tsai MY; 1994, Maurizio Ferrari; 1996). It has been shown that a single-base difference at or near the terminal 3′ base of a primer affects PCR reaction results more dramatically than those single- base differences located internally or at 5′ end (Lindeman R; 1991). Using primers with one or more base- difference near the 3′ end, allele specific amplification (ASA) has been developed (Okayama H; 1989) for the detection of mutations in genetic diseases (Masato Tanigawa; 2000). In this study, we have evaluated the effect on PCR, of one different (**C-G**) base located at the 7^{th} position from 3′–terminal end of primers, where one with the **C** base represents a Human DNA sequence from clone RP11-422P24 on chromosome 1 (between 1pter and 1qter) which contains the 5' UTR of the gene for transcription repressor p66beta component of the MeCP1 complex (P66beta).This transcription repressor gene that codes for a component of the MeCP1 protein complex, represses transcription through preferential binding, remodeling and deacetylation of methylated nucleosomes complex (Brackertz M . et al;2002, Brackertz M et al; 2006,Okkelman IA et al ; 2014). Also known as CTG-B1(aliases; KIAA1150, D1S961E, CTG-B1-1, CTG-B1-2, CTG-B1-1/CTG-B1-2), this gene has been for long time considered as strong candidate for abnormal expression in case of people suffering from schizophrenia as it has been reported in some population to have abnormal number of trinucleotide (CAG) in its sequence though has not been established as yet. Studies on Caucasian population and Japanese populations have been reported with positive and negative observations (Ohara K etal; 1997).

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II.METHODOLOGY

DNA ISOLATION:

High-salt, Phenol-Chloroform method [Miller SA, 1988] was used to isolate genomic DNA from whole blood (5ml) with ethylenediamine tetra acetic acid (EDTA) as anticoagulant. The nuclei of white blood cells were isolated after lysis of cells with a non-ionic detergent. The isolated cell nuclei were suspended in a buffer containing an ionic detergent (SDS) and proteinase K that digests the proteins associated with chromosomal DNA. All the proteins were precipitated using high salt concentration (salting out) followed by three washes of phenol, phenol-chloroform and isoamyl-chloroform and the DNA in the supernatant was recovered by precipitation with ethanol. The amount of DNA obtained was around 5–10 μg per 0.5 ml whole blood.

DNA amplification by Polymerase Chain Reaction:

Taking as standard for study, a small region (126 bp) of human DNA gene sequence which contains 5' UTR of the gene for transcription repressor p66beta component of the MeCP1 complex was amplified using a pair of primer which were either completely complementary to this transcription repressor gene or had a single substituted difference at the 3′–end of one of the strand of the primer used. Genomic DNA was amplified in 25 μl reaction volumes with:

10X PCR buffer	2.5 □l
(100 mM Tris Hcl pH 8.8; 500 mM KCl; 0.8% Nonidet P40)	
Magnesium chloride 25 mM	1.5□l
dNTP mix 1.25 mM	4.0 □l
sense primer	~10picomoles
antisense primer	~10picomoles
Taq DNA polymerase	0.25units
Genomic DNA	1□l

Volume was made upto 25 □l with triple distilled water. Equal volume of mineral oil was added to layer the reaction on upper surface. All amplifications were started by the addition of the Taq DNA polymerase at 96˚C (hot start) and continued for 32 cycles in a DNA thermal cycler (Perkin Elmer Cetus.) Each cycle of amplification contained the following steps: DNA denaturation: 1 minute at 96˚C, primer annealing: 1 minute at 50˚-58˚C, and primer extension: 1 minute at 72˚C, with finally extension at 72˚C for 7 minutes. The PCR

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products were separated by electrophoresis on 2.0% agarose gels and detected by staining with ethidium bromide (0.5 µg/ml) for 15 minutes in electrophoresis buffer TAE (40 mM tris acetate pH 8.0 and 1mM EDTA). Pictures of the gels over UV gel documentation (UVi DOC, v.99) were taken.

Synthesis of DNA primers

Two sets of oligonucleotide primers were synthesised (Microsynth, GmbH). One set **completely matched** the template DNA **(P66beta)**

SA58 (F) 5'CTT GAT GTG CTG A**C**A CCT GC 3'

SA51(R) 5'AAG CTC CGG AAT GTT GTC C3'

While the other had an upstream primer with a **substituted (C-G)** at 7th position from its 3′–end.

SA52 (F) 5'CTT GAT GTG CTG A**G**A CCT GC 3'

SA51(R) 5' AAG CTC CGG AAT GTT GTC C 3'

(* different base shown in red alphabets).

PCR reactions applied

Primer	Pre-PCR Denaturation	PCR reaction	Post-PCR Extension	Number of cycles	Reaction Result
With C base (SA58-SA51)					
1	96^{O}C-5mins.	96^{O}C-1 min. 50^{O}C-30 secs. 72^{O}C -1min.	72^{O}C-7 mins.	32	Negative
2	96^{O}C-5 mins.	96^{O}C -1min. 55^{O}C-1 mins. 72^{O}C-1 mins.	72 ^{O}C-7 mins.	32	**Positive**
3	96^{O}C- 5mins.	96^{O}C-1 mins. 58^{O}C-30 secs. 72^{O}C-1min.	72 ^{O}C-7 mins.	32	Negative
4	96^{O}C –5 mins.	96^{O}C -1mins. 60^{O}C-30 secs. 72^{O}C-2 mins.	72 ^{O}C-7 mins.	32	Negative

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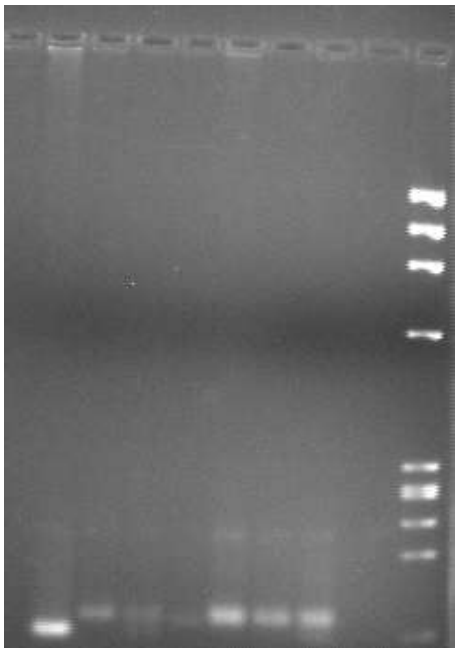
With G base (SA52-SA51)					
1	96^{O}C-5 mins.	96^{O}C-1 min. 50^{O}C-30 secs. 72^{O}C -1min.	72^{O}C -7mins.	32	**Positive**
2	96^{O}C-5 mins.	96^{O}C -1min. 55^{O}C-1 mins. 72^{O}C-1 mins.	72^{O}C-7mins.	32	Negative
3	96^{O}C-5 mins.	96^{O}C-1 mins. 58^{O}C-30 secs. 72^{O}C-1min.	72^{O}C-7mins.	32	Negative
4	96^{O}C-5 mins.	96^{O}C -1mins. 60^{O}C-30 secs. 72^{O}C-2 mins.	72^{O}C-7mins.	32	Negative

1 2 3 4 5 6 7 M

IA

1 2 3 4 5 6 7 M



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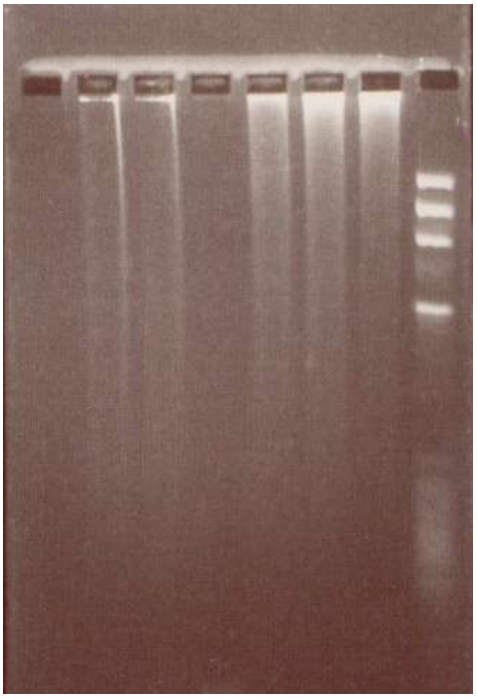
1 2 3 4 5 6 7 M

IC

1 2 3 4 5 6 7 M



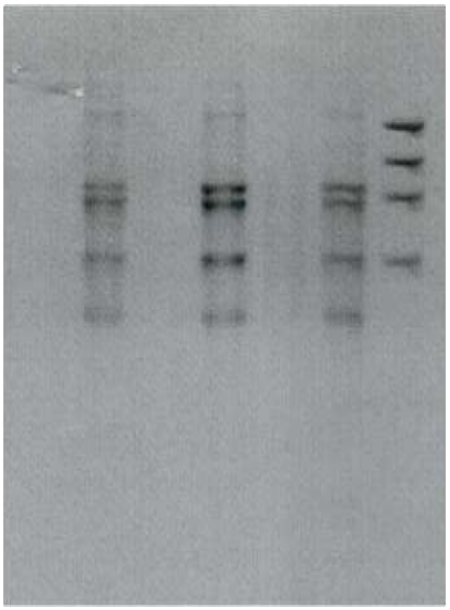
ID

Figure I : PCR product of genomic DNA at different temperatures(Ta^0C) with primer SA 58-51(with C base at 7' position)

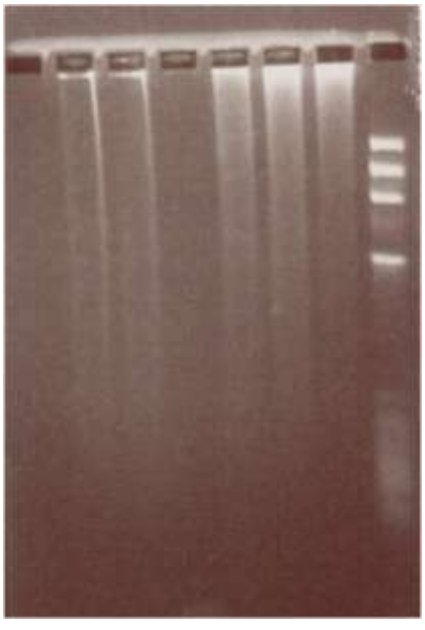
IA: Ta^0C 50^0C; IB : Ta^0C 55^0C; IC: Ta^0C 58^0C; ID: Ta^0C 60^0C

M:marker- φx174; lanes (1-7& 1-9) genomic DNA

1 2 3 4 5 6 7 8 9 M



1 2 3 4 5 6 7 M



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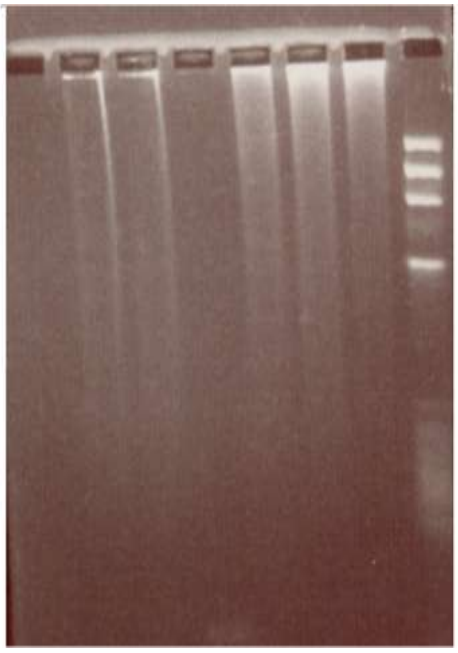
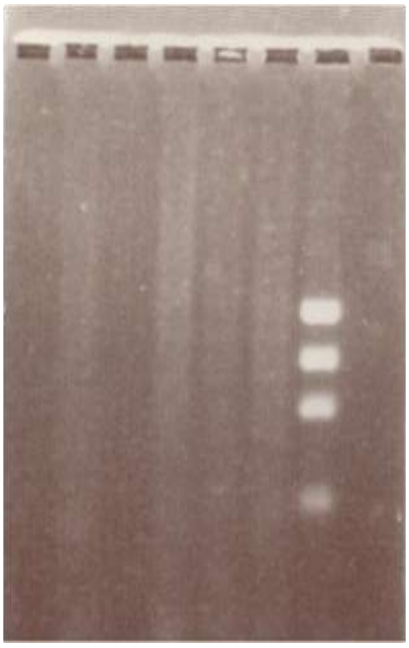
IIA

1 2 3 4 5 6 7 M



IIB

1 2 3 4 5 6 7 M



IIC **IID**

Figure II : PCR product of genomic DNA at different temperatures(Ta^0C) with primer SA 52-51(with G base at 7'position)

II A: Ta^0C 50^0C; II B : Ta^0C 55^0C; II C: Ta^0C 58^0C; II D: Ta^0C 60^0C

M:marker- φX174; lanes (1-3,1-6 & 1-7) Human genomic DNA

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Table-1

Molecular weight of PCR products at T^0C 55 for primer with C base at 7'position

M	L3	L4	L5	L6	L7	L8	L9
13.53	1.318	1.333	1.349	1.349	1.365	1.241	1.241
10.78							
8.72							
6.02							
3.1							
2.81							
2.71							
2.34							
1.94							
1.18							
0.72							

***** **M :: Marker X174 Mol.wt. values**

L3 - L9:: Genomic DNA Mol.wt. values

Table-2

Molecular weight of PCR products at T^0C 50 for primer with G base at 7' position

M	L2	L3	L4
13.53	9.175	9.136	9.254
10.78	8.537	8.464	8.5
8.72	6.116	6.116	6.149
6.02	3.725	3.657	3.792
3.10			
2.81			
2.71			
2.34			
1.94			
1.18			
0.72			

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****** **M :: Marker X174 Mol.wt. values**

L2,L3,L4

:: Genomic DNA Mol.wt. values

III.RESULTS

The primer SA58-SA51 (with C base) used in this work specified the amplification of a 126 bp region of the human P66beta gene.Thus is taken as standard primer for identification of the PCR amplified product. Before studying the effect of difference at the position from 3′–end of primer, an optimization for $MgCl_2$ was carried out since any PCR amplification is known to be very sensitive to magnesium ion concentration (Saiki RK, 1985). Using the standard primers (primer with C base), it was found that a detectable 126 bp PCR product was amplified at different Mg^{+2} ion concentrations ranging from 0.5 to 2.5 mM in 0.5 mM increments (data not shown). However, above 2.0 mM concentration, there were too many nonspecific products, and furthermore, the desired 126 bp product decreased in quantity with increased Mg^{+2} ion concentration. The best amplification of the 126 bp product was achieved at 2.0 mM $MgCl_2$ concentration. To study the effect of C/G substitution, we attempted to amplify the 126 bp DNA fragment at different annealing temperatures from 50^{o}C to 60^{o}C using a "normal" downstream primer (SA51) with all bases at proper positions together with upstream primer (SA52) containing a single substitution at the 7th position from 3′–end of sense (forward) primer leading to difference. Having a C residue in the primer strand at the corresponding position, this primer normally formed 20 normal base pairs with the template, but in this case, resulted in a difference of C/G at the 7th position from 3′ terminal end. Figure 1 shows some representative results obtained with different primers at 50^{o}C and 60^{o}C. Of all the annealing temperatures applied- 50^{o}C, 55^{o}C, 58^{o}C, 60^{o}C, when standard primer pair (primer with C base) was used, the desired 126 bp DNA fragment was amplified satisfactorily without any detectable non-specific products at 55^{o}C (Fig. I.B, Table 1). However, the results obtained at other annealing temperatures were different. 58^{o}C and 60^{o}C showed the negative result (smear) (Fig. I; C, D) while as at 50^{o}C, annealing temperature showed multiple non-specific products (Fig.I; A). The primer with the C/G substitution (Figure II) showed no detectable 126 bp product at any of the temperatures-55^{o}C, 58^{o}C, 60^{o}C (Fig.II, B, C, D) but fairly distinct larger PCR product (s) than 126 bp at 50^{o}C respectively (Fig.II; A, Table 2).

IV.DISCUSSION

We studied the effect of single base substitution (C/G) on amplification of DNA segment and therein the change in the result on the identification of the amplified product. The different G residue was located at the 7th position of P66beta gene from the 3′–end of the primer. Our results showed the absolute contradictory results due to a single base replacement. As expected of the primer the synthesis of 126bp specific PCR product expected of SA58-51 is

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extremely poor and resulted in the production of specific PCR fragment much larger in molecular weight (s), detectable by staining the gel with ethidium bromide. The results obtained with the difference (G base; Figure. II) at different annealing temperatures tested (50 to 60ºC) showed no specific product was detectable at all at 55ºC, 58ºC and 60ºC (Fig.II; B, C, D) of the tested annealing temperatures. The C/G difference, however, produced a fairly strong and distinct PCR products at 50ºC (II, A) sized in the range of 950- 400 bp (Table 2), not produced at all at higher annealing temperatures (Figure II). Various groups have already studied the priming efficiency of single- base difference primers. Our result with C/G difference disagrees with that of Kwok (Kwok S, 1990) who showed that a single difference of T/T, T/C or T/G at 3′–end had no effect on the priming efficiency of their primers. Newton (Newton CR, 1989) reported that a single C/G difference had very little effect on the priming efficiency of their primers, while we couldn't get any detectable specific PCR product at 55ºC, 58ºC or 60ºC. However, it is difficult to make a direct comparison of the results reported with various 3′ single-base difference primers, due to differences in the substitution, PCR amplification conditions, especially the length and concentration of primers. In some studies, the primers were too short (12–16 bases) (u DY, 1989)and hence a 3′–single base difference may have more dramatic effects both on the stability of primer-template duplex and the extension of one- base difference primer by DNA polymerase. On the other hand, if the primers are too long, like 30 bases used by Ehlen and Dubeau (Ehlen T) the effect of 3′–difference on PCR may be minimal. The primers used in this work were intermediate in size (20 bases long), a size commonly used by many research groups. More so importantly, the single-base difference at any position in the primer does not nullify the presence of sequences in the overall DNA assessment, as in our study the (C/G) difference at the 7^{th} position of primer which represents P66beta gene from the 3′–end resulted in distinct larger PCR product (s) than the expected 126 bp. This can lead to finding of specific sequences which might play a vital diagnostic role in the identification of diseases like neuropsychiatric diseases as schizophrenia where molecular aspect as sequence expansion has been said to play a role in disease onset. A single base difference leads to identification of other DNA products present in human genome as is evident from Fig. The product of 126 bp is observed at 55^{o}C in standard primer SA58-51but the absolute absence of any product at same annealing temperature SA52-51 proves that a single base substitution can lead to misdiagnosis of a disease if PCR amplification is set as tool for identification.

V.CONCLUSION

In conclusion, our upstream primers resulting in a G/C substitution difference at the 3′– end with template DNA showed wide difference in amplification products. The identification of sequences as outcome of a single-base difference cannot be foregone and shall be studied extensively.


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