Meta Gene 2 (2014) 106–113

Contents lists available at ScienceDirect

Meta Gene

Application of a reliable and rapid polymerase chain reaction based method in the diagnosis of myotonic dystrophy type 1 (DM1) in India ☆

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ARTICLE INFO

Article history:
Received 26 September 2013
Revised 26 November 2013
Accepted 2 December 2013
Available online 15 January 2014

Keyword:
Triplet repeat disorder
Myotonic dystrophy type 1 (DM1)
CTG repeat
TPPCR

ABSTRACT

DM1 is caused by CTG repeat expansion in the 3′-UTR of the *DMPK* gene. DM1 patients have expansions of greater than 50 repeats and up to many thousands. The intention of the present study is the establishment of reliable and rapid polymerase chain reaction methodology in early screening of DM1 patients and their family members. PCR followed by TP-PCR was assessed for screening of 27 cases (from 26 families) and 75 family members and 300 control samples. All patients had CTG repeat expansion while forty seven (63%) and twenty eight (37%), out of seventy five family members were heterozygous and homozygous respectively. Similarly, two hundred thirty (76.77%) and seventy (23.33%), out of three hundred control subjects were heterozygous and homozygous respectively and the number of repeats varied from 5 to 35. Thirteen complete family screenings were done. Thus, TP-PCR is a reliable and rapid molecular technique for the detection of CTG repeat expansion in DM1.

Introduction

Myotonic dystrophy (DM) is a chronic, slowly progressing, highly variable, inherited multisystem autosomal-dominant and triplet repeat disorder and it is characterized by a marked intrafamilial and

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http://dx.doi.org/10.1016/j.mgene.2013.12.001

interfamilial clinical variability. There are two types of DM, namely myotonic dystrophy types 1 and 2 (DM1 and DM2) which are caused by mutation in two different genes *DMPK* (*Dystrophia myotonica protein kinase*) and cellular nucleic acid-binding protein i.e. *CNBP* (previously known as *ZNF9* i.e. *Zinc finger protein* (Table S1)). It affects approximately 1 in 7500 individual worldwide (Harper, 1989). The incidence for DM2 seems to be much lower than for DM1 and appears to be population dependent, reaching a higher incidence in Germany and Finland (Suominen et al., 2011). The disorder shows a phenomenon of genetic anticipation in which affected individuals in succeeding generations have an earlier age of onset and a more severe clinical course (Howeler et al., 1989) due to the expansion of the repeat number during gametogenesis. Repeat contraction events occur 4.2 to 6.4% of the time in DM1 families and contraction in a relative increases the probability of another contraction in the family (Ashizawa et al., 1994). The correlation between CTG repeat size observed in one tissue (e.g. blood) often does not match the severity of the disease and the CTG repeat size in other organs (e.g. muscle).

On the basis of clinical symptoms DM1 was classified into four different subtypes: (i) mild, (ii) classical, (iii) juvenile and (iv) congenital types: (i) mild DM1: mildly symptomatic patients have premature cataracts and baldness as the sole clinical features. A late-onset myopathy develops and myotonia was only detectable by electromyography. Cardiac conduction abnormalities arise and result in a shorter life span (Kamsteeg et al., 2012), (ii) classical or adult-onset DM1: The age of onset is typically in the second or third decade of life. The most frequent symptoms are distal weakness, involving the long finger flexors of the arms and the dorsiflexors of the legs, leading to symptoms relating to the strength of hand grasping and an increased incidence of stumbling. In addition patients have cataracts, baldness and cardiac conduction abnormalities (Kamsteeg et al., 2012), (iii) juvenile DM1: this form resembles the classical form of myotonic dystrophy. However, it is more clearly associated with cognitive and behavioral abnormalities (Kamsteeg et al., 2012) and (iv) congenital DM1: Polyhydramnios and poor fetal movements precede the birth of an infant with congenital DM1 (Kamsteeg et al., 2012). The affected parent is nearly always the mother and congenital DM1 occurs in a quarter of offspring of affected DM1 mothers (Harper, 2001). The DM1 mutation involves an expanded trinucleotide repeat (CTG) in the *DMPK* (gene in the 3′-untranslated region) (Buxton et al., 1992; Fu et al., 1992; Mahadevan et al., 1992) and varies in the normal population from 5 to 35 and >50 repeat is associated with DM1 severity (Kamsteeg et al., 2012). The *DMPK* gene is ~14 kb and encodes 2.3 kb of mRNA with 15 exons and a protein (cAMP-dependent serine–threonine kinase) of 624 amino acids (Mahadevan et al., 1993; Shaw et al., 1993). Offspring of an individual with an expanded allele have a 50% chance of inheriting the mutant allele.

The genetic testing of DM1 plays a critical role in characterizing them and directing clinical person to cater them for the appropriate therapy and management. Clinically, myotonic dystrophy is diagnosed by the elevated level of muscle enzyme SCK (serum creatinine kinase), characteristic pattern of electromyography peaks, nerve conduction velocity (NCV) and various other parameters. PCR–RFLP (Sermon et al., 1997; Shaw et al., 1985) followed by southern blotting (Goossens et al., 2008; Sermon et al., 1998, 2001) is used for the detection of CTG repeats. The TP-PCR (Triplet Primed-Polymerase Chain Reaction) technique was developed by Warner et al. (1996) for screening the CAG repeat expansion in myotonic dystrophy and the utility of TP-PCR in DM1 was shown by Radvansky et al. (2011). It provides characteristic peak pattern which confirms the existence of triplet repeats in the *DMPK* gene (Warner et al., 1996).

The intention of the present study is to establish the conventional PCR followed by TP-PCR methodology in early screening of DM1 patient and family members in Indian setup. As per our knowledge, this is the first study that engages TP-PCR as a method of screening for DM1 in India.

Materials and methods

In this study 27 patients from 26 families reported to the Departments of Neurology and Genetics OPD, SGPGI, Lucknow were recruited along with their family members (total 75) and control samples (total 300), and before enrolment informed consent was obtained from the subjects. 2 ml of blood collected in EDTA vials served as a source for DNA extraction and it was accomplished by Qiagen DNA extraction kit. The quality and quantity of DNA were assured by agarose gel electrophoresis and spectrophotometry.

Amplification of CTG repeats

Polymerase chain reaction

Amplification of DMPK triplet repeats was carried out using 100 ng of DNA in 25 μl reaction using 2 pairs of primers having conc. 20 pmol each (Hamzi et al., 2010). Hot start procedure was followed after denaturation at 95 °C for 10 min followed by 35 cycles of 95 °C for 1 min, 64 °C for 1.5 min, and 72 °C for 2 min with a final extension of 72 °C for 5 min. The final products were analyzed on 3% agarose gel.

Triplet Primed-Polymerase Chain Reaction (TP-PCR)

TP-PCR assay was executed with the primers described by Warner et al. (1996). TP-PCR assay was carried out in a reaction volume of 25 μl with P1, P2 and P3 (flanking primer) and P4 (internal primer) primers in the concentration of 10 pmol each along with 50 ng of genomic DNA, 1 U of Taq Polymerase and 200 μM of dNTPs. The temperature profile adopted in TP-PCR cycling was 4 min at 94 °C followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and 1 cycle of 72 °C for 10 min. The final products were analyzed on 2% agarose gel and segment analysis was done by the ABI-310 genetic analyzer by the use of polymer (POP-4), Liz-500 and Hi-di formamide.

Results

Conventional PCR followed by TP-PCR was assessed for screening of 27 cases (from 26 families) and 75 family members and 300 control samples. In a family (fam. 14) both son and his mother were clinically suspected for DM1 and was also confirmed by PCR followed by TP-PCR with the expanded repeat of CTG. Other clinically suspected fourteen cases were also subjected for the same methodology for DM1 confirmation. Clinical profile of all 27 myotonic dystrophic patients is listed in Table 1. Most of the patients

Table 1
Clinical feature of 26 DM1 patients.

Patient/sex	FH	Age at onset	Duration	MW	JTW	FW	HS	EMG	NCV	SCK (U/L)
1/M	N	28	2	Y	Y	Y	Y	+	+	156
2/M	N	23	6	Y	N	Y	N	+	+	272
3/F	N	43	2	Y	Y	Y	Y	+	+	88
4/M	N	27	4	Y	Y	Y	Y	+	+	95
5/M	N	25	5	Y	Y	Y	Y	+	+	124
6/M	N	20	3	Y	Y	Y	Y	+	+	220
7/M	Y	13	4	Y	Y	Y	Y	+	+	475
8/M	N	36	4	Y	Y	N	N	+	+	73
9/F	N	30	7	Y	Y	Y	Y	+	+	430
10/M	N	39	1	Y	Y	Y	N	+	+	1346
11/M	N	10	15	Y	Y	Y	N	+	+	225
12/M	N	29	5	Y	Y	Y	Y	+	+	256
13/M	N	34	2	Y	Y	Y	Y	+	+	1437
14/M	Y	9	11	Y	Y	Y	Y	+	+	325
14m/F	Y	37	5	Y	Y	Y	Y	+	+	255
15/F	N	40	3	Y	Y	Y	Y	+	+	54
16/M	N	10	15	Y	Y	Y	N	+	+	62
17/M	N	5	22	Y	Y	Y	Y	+	+	68
18/M	N	17	18	Y	Y	Y	Y	+	+	194
19/M	N	49	3	Y	Y	Y	N	+	+	1700
20/F	N	39	1	Y	Y	Y	Y	+	+	222
21/M	N	22	5	Y	Y	Y	Y	+	+	412
22/M	N	16	13	Y	Y	Y	Y	+	+	285
23/M	N	26	5	Y	Y	Y	N	+	+	198
24/F	N	7	2	Y	Y	Y	Y	+	+	486
25/M	N	47	10	Y	Y	Y	Y	+	+	163
26/M	M	49	2	Y	Y	Y	Y	+	+	335

FH, family history; MW, muscle wasting; JTW, jaw and temporal wasting; FW, facial weakness; HS, hypersomnia; EMG, electromyography; NCV, nerve conduction velocity; SCK, serum creatinine kinase; 14m, mother of the patient 14; M, male; F, female; N, no; Y, yes; +, positive.

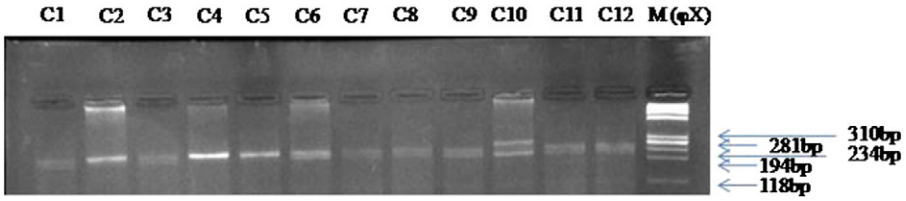

Fig. 1. Control of the *DMPK* amplification on a 3% agarose gel (healthy subjects) M: φx DNA ladder; C: healthy subjects; —C1, C3, C6, C7, C8, C9, C10, C11 and C12 are heterozygous presenting 2 bands; C2, C4 and C5 are homozygous presenting only one band.

have muscle wasting, jaw and temporal wasting, facial weakness and hypersomnia during presentation of the disease (Table 1). In TP-PCR analysis control subject showed no expansion of CTG repeat allele (Fig. 5a) while patient had expanded CTG repeat allele (Fig. 5b) and proband's family member had either normal (Fig. 5c) or permutated or pathogenic CTG repeat allele.

Molecular results

Normal subjects

The range of PCR products of the normal *DMPK* allele varied from 205 to 310 bp for a number of repeats from 5 to 35. In three hundred subjects from the general population, we found a size of bands less than 310 bp (<37 CTG repeat number), two bands for the heterozygous (C1, C3, C6, C7, C8, C9, C10, C11 and C12) and one intense band for the homozygous (C2, C4 and C5). Out of three hundred, two hundred thirty (76.77%) and seventy (23.33%) were heterozygous and homozygous respectively. Fig. 1 showed amplification products from the *DMPK* loci analyzed on a 3% agarose gel for the control groups.

Patients and their family members

All the twenty seven patients and seventy five family members were screened for *DMPK* mutations by PCR approach. Fig. 2 represented result for DM1 patients and their family members. Out of seventy five DM1 family members, forty seven (63%) and twenty eight (37%) were heterozygous and homozygous respectively. TP-PCR was done for confirmation and the results were similar as presented in Table 2. All DM1 patients had one expanded CTG repeat *DMPK* allele while the other normal CTG repeat *DMPK* allele in four patients had 5 CTG repeats, three patients had 9 CTG repeats, sixteen patients had 12 CTG repeats and the remaining four patients had 13 CTG repeats (Table 2).

The complete family screening was performed in thirteen cases (families 2, 5, 6, 7, 8, 10, 12, 15 to 20). The age of patient at the onset of disease and various clinical symptoms are listed in Table 1. In the present study we are illustrating the profile of the first six families. Case one (DM1 family 2): Patient had CTG repeat expansion and family members had normal for CTG repeat. Case two (DM1 family 5): Patient had CTG repeat expansion and the father was normal for CTG repeat while the mother died before the testing

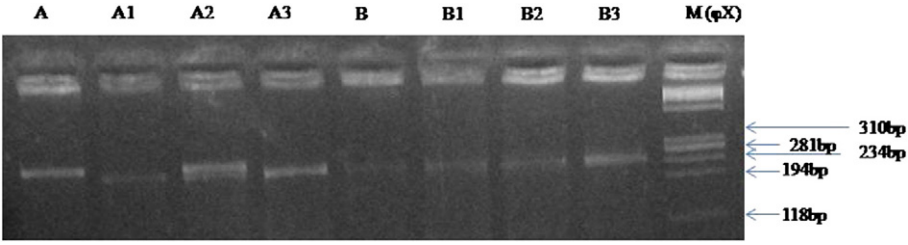

Fig. 2. The *DMPK* amplification of DM1 patients and their family members on a 3% agarose gel. M: φx DNA ladder; A and B are DM1 patient while A1 to A3 and B1 to B3 are respective family members of DM1 patients A and B. Only A2 is heterozygous presenting 2 bands while A, A1, A3, B, B1, B2 and B3 are homozygous presenting only one band.

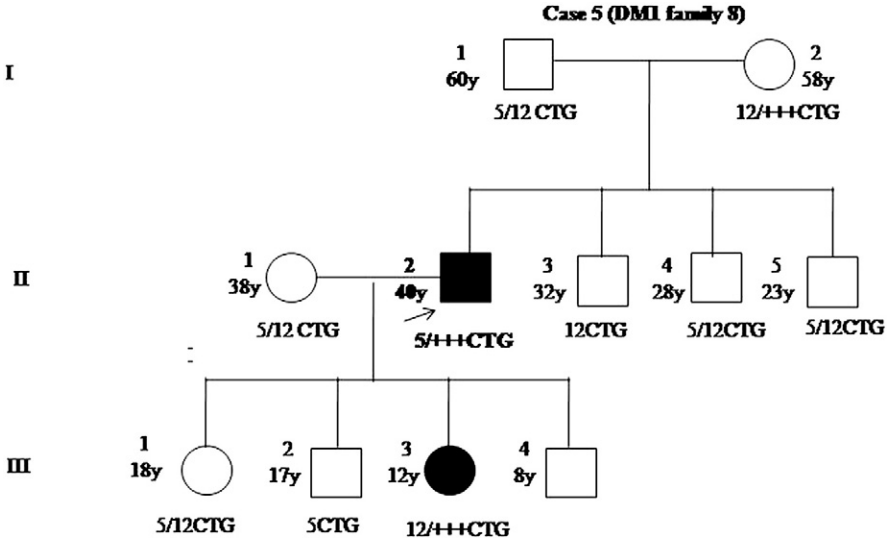

Fig. 3. Case five (DM1 family 8) screening: Patient (II.2) and his daughter (III.3) had pathogenic CTG repeat. Except proband's mother (I.2), all family members were normal for CTG repeats.

of the patient for the disease and patient had no other siblings. Case three (DM1 family 6): Patient had CTG repeat expansion and parents died before the testing of the patient and patient had no other siblings. Case four (DM1 family 7): Patient had CTG repeat expansion while all family members had normal CTG repeats. Case five (DM1 family 8): Patient (Proband) and his daughter (III.3) had disease associated CTG repeat

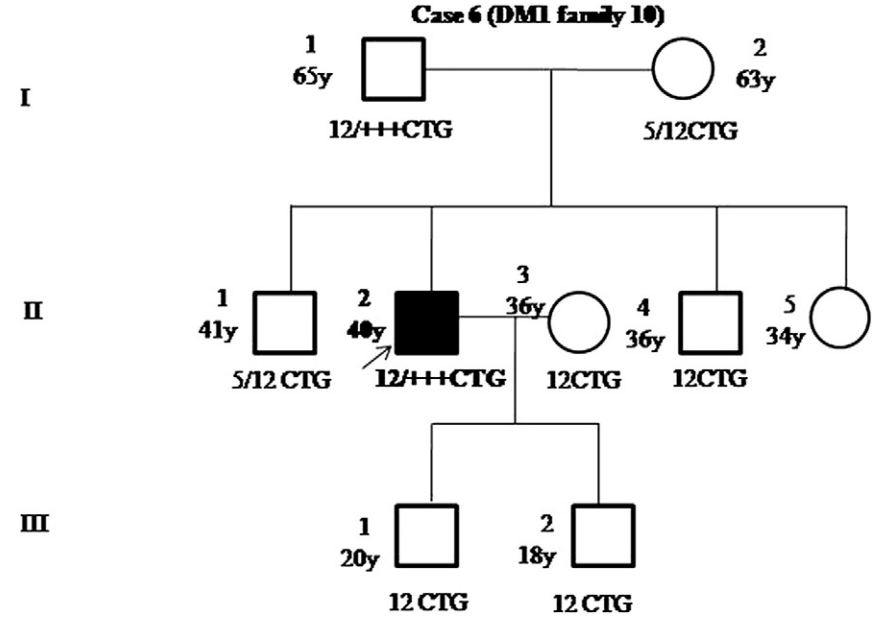

Fig. 4. Case six (DM1 family 10) screening: The DM1 patient (II.2) had one normal (12CTG) and other pathogenic CTG repeat allele. All family members, except proband's father (I.1), were normal for repeat.

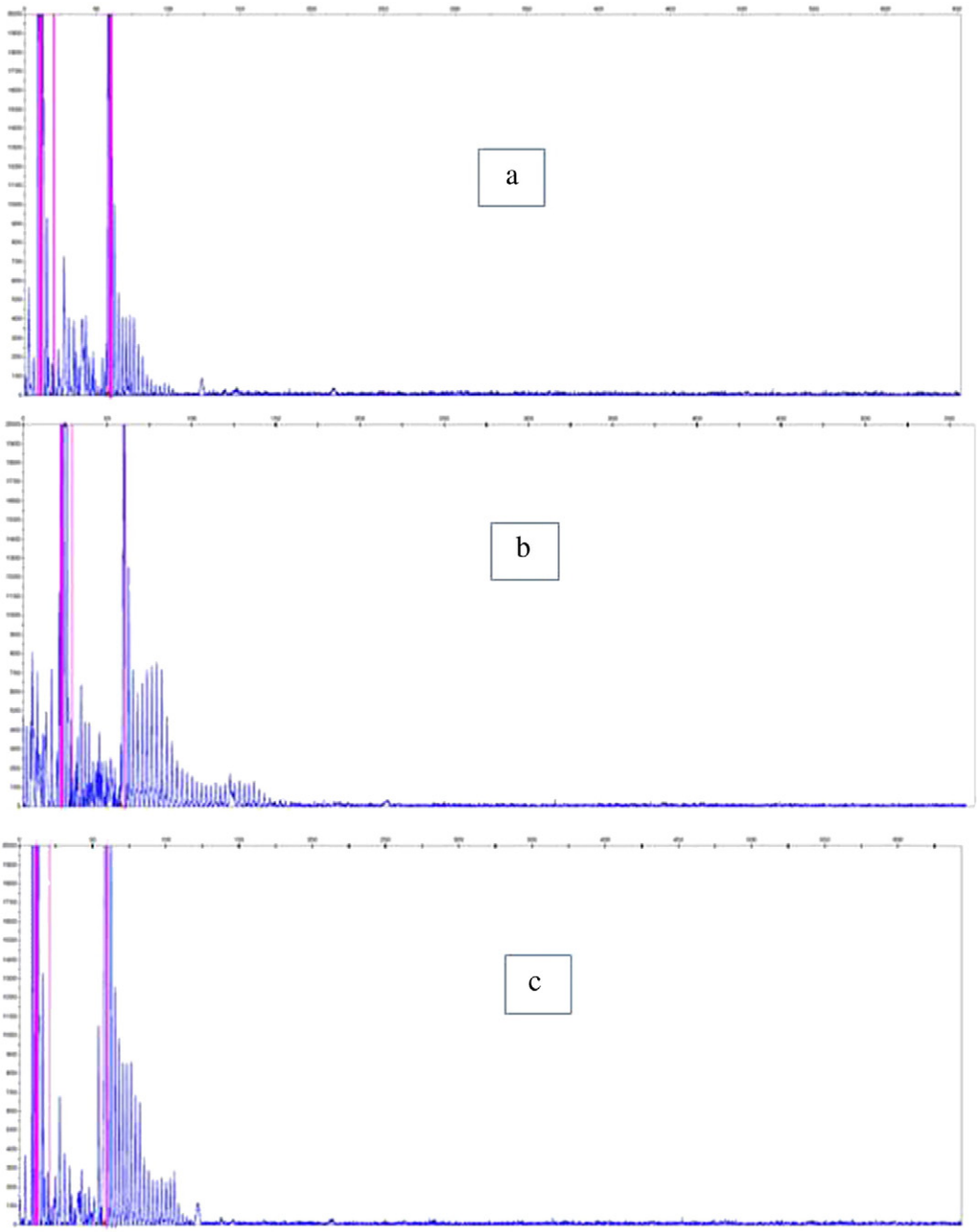

Fig. 5. TP-PCR product Gene scan analysis of (a) control subject that had no CTG repeat expansion, (b) a patient that had expanded CTG repeat allele and (c) a normal individual of a DM1 family member. Horizontal and vertical scales indicate size in base pairs and fluorescent intensity of the expanded alleles respectively.

allele. Proband's mother had one normal and one permutated CTG repeat allele and the CTG repeat pattern of the remaining family members had been illustrated in Fig. 3. Case six (DM1 family 10): Proband's father had one normal while other permutated allele. The DM1 patient (II.2) had one normal (12CTG) and other

Table 2
PCR and TP-PCR results of all 26 DM1 patients.

DM1 family S.N.	Sample analyzed	PCR result (band)	TP-PCR result
1	P	1	12CTG/+++
2	P	1	12CTG/+++
3	P	1	12CTG/+++
4	P	1	12CTG/+++
5	P	1	12CTG/+++
6	P	1	12CTG/+++
7	P	1	5CTG/+++
8	P	1	5CTG/+++
9	P	1	12CTG/+++
10	P	1	12CTG/+++
11	P	1	9CTG/+++
12	P	1	9CTG/+++
13	P	1	12CTG/+++
14	P	1	12CTG/+++
14	M	1	12CTG/+++
15	P	1	12CTG/+++
16	P	1	5CTG/+++
17	P	1	13CTG/+++
18	P	1	13CTG/+++
19	P	1	13CTG/+++
20	P	1	13CTG/+++
21	P	1	9CTG/+++
22	P	1	12CTG/+++
23	P	1	12CTG/+++
24	P	1	12CTG/+++
25	P	1	5CTG/+++
26	P	1	12CTG/+++

P, DM1 patient; M, mother of the patient of the DM1 family fourteen.

pathogenic CTG repeat allele. The pattern of CTG repeat allele in remaining family members had been shown in Fig. 4.

Discussion

Myotonic dystrophy (DM) is a common neuromuscular disorder comprising at least two genetically different forms. DM1 is caused by the expansion of a (CTG)(n) repeat in the *DMPK* gene, while DM2 is caused by the expansion of a (CCTG)(n) part of a complex repetitive motif (TG)(n)(TCTG)(n)(CCTG)(n) in the *CNBP* gene (Radvansky et al., 2011a, 2011b). DM1 is also a kind of progressive multisystemic autosomal dominant disorder with phenomena of anticipation. It denotes progressively earlier onset of the disease within successive generations. The disabilities are substantial and therefore early detection is mandatory for reproductive counseling of families in which the DM1 has been observed as they have 50% chances of each pregnancy to have an affected child.

In molecular methods, southern blotting, although used widely has a long laboratory turnaround time, is relatively expensive and got hazards associated with the use of radioisotopes (Khajavi et al., 2001). So, PCR method is a rapid and cheap method for initial screening. The best protocol would be to perform PCR for initial screening followed by TP-PCR and southern blots for confirmation. The standard PCR allows us to amplify the normal allele (<310 bp). We were able to obtain either 2 bands of distinct size <310 bp or an intense band which corresponds to two overlapping bands of the same size. In patients we have obtained only one band corresponding the normal allele. PCR could not amplify the pathological fragment that causes its larger size (owing to the higher no. of CTG repeats). The TP-PCR can detect the presence of long allele size without determining the total size of the expansion or the exact no. of CTG repeat (Madhumita et al., 2006).

The advancement in present PCR technique and DNA fragment analysis method established that TP-PCR could be successfully used for the identification of repeat size in myotonic dystrophy and other

repeat expansion disorder following similar mutational mechanism. It can be used successfully in place of southern blot which is radioactive and time consuming technique. Only the seemingly positive cases should be confirmed by the southern blotting method. This TP-PCR technique could provide a useful method for screening myotonic dystrophy especially in instances where presymptomatic and prenatal diagnosis is required.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2013.12.001.

Acknowledgments

Authors are thankful to Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow for providing infrastructure facility in the department. Ashok Kumar is thankful to DBT, New Delhi for his fellowship. All the authors declare that they have no conflict of interest.

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