



Draft Best Practice Guidelines for Molecular Analysis of Hereditary Motor and Sensory Neuropathies

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Disclaimer

These Guidelines are based, in most cases, on the reports drawn up by the chairs of the disease-based workshops run by EMQN and the CMGS. These workshops are generally convened to address specific technical or interpretative problems identified by the QA scheme. In many cases, the authors have gone to considerable trouble to collate useful data and references to supplement their reports. However, the Guidelines are not, and were never intended to be, a complete primer or "how-to" guide for molecular genetic diagnosis of these disorders. The information provided on these pages is intended for chapter authors, QA committee members and other interested persons. All the guidelines are at a draft stage, and must not be used until formally published. Neither the Editor, the European Molecular Genetics Quality Network, the Clinical Molecular Genetics Society, the UK Molecular Genetics EQA Steering Committee nor the British Society for Human Genetics assumes any responsibility for the accuracy of, or for errors or omissions in, these Guidelines.

Nomenclature and gene ID

See table 1

Description of the disease

Charcot-Marie-Tooth disease (CMT) is the most common inherited peripheral neuropathy. After the first description of the disease in 1886, it became clear that CMT was not one single disease entity, but was clinically and genetically heterogeneous. In 1968, Dyck and Lambert introduced a classification of peripheral neuropathies based on genetic, electrophysiological and neuropathological criteria. Hereditary motor and sensory neuropathy type I (HMSN I, CMT1) is characterized by severely slowed motor and sensory nerve conduction velocities (NCV) and signs of de- and remyelination on sural nerve biopsy. The disease is mostly inherited as an autosomal dominant trait, but also X-linked, autosomal recessive and isolated cases are found. It is the most common inherited peripheral neuropathy with a prevalence of 10 - 40 / 100 000. Most frequently a 1.5 Mb tandem duplication comprising the *PMP22* gene is observed, but also mutations of the peripheral myelin protein 22 (*PMP22*, 17p11.2), myelin protein zero (*MPZ*, *PO*, 1q22-23), Connexin32 (*Cx32*, Xq13.1) and the early growth response 2 (*EGR2*, 10q21.1-22.1) genes have been

reported. All except *EGR2*, a transcription factor, represent transmembrane proteins located in the myelin sheath of peripheral nerves. HMSN II or CMT2 is characterized by normal or slightly reduced motor and sensory NCVs. It is genetically heterogeneous with autosomal dominant or recessive inheritance. HMSN III or Dejerine-Sottas syndrome (DSS) is a very severe neuropathy with extremely reduced NCV and early onset age. Patients with hereditary neuropathy with liability to pressure palsies (HNPP) usually develop a mononeuropathy after minor trauma. On careful examination, some patients show a more generalized peripheral neuropathy. HNPP is inherited as an autosomal dominant trait, most frequently a deletion reciprocal to the CMT1A duplication is observed.

Common reasons for referral

Although the phenotype may vary over a wide range there are some common reasons for patients to consult clinicians for diagnosis over their disease. Most frequently gait disturbances and slowly progressive distal weakness in the feet and/or hands, but also recurrent palsies or foot deformities include reasons for a clinical consultation. Testing of NCV/EMG, occasionally a sural nerve biopsy and reports of a family history frequently result in a genetic consultation. The inherited neuropathies need to be carefully distinguished from the many causes of acquired (non-genetic) neuropathies, including immune mediated, toxic, infectious and deficiency states.

Approaches and protocols

A wide range of techniques is available for molecular diagnostic testing in CMT. These guidelines should not be understood as prescriptive, but to highlight advantages and pitfalls for some of the techniques.

Most laboratories still carry out a Southern hybridisation based on dosage differences of the restriction fragment length polymorphisms, other techniques like FISH or PCR based methods are upcoming.



Clinical examination

The motor system and/or the sensory system can be involved in neuropathies. Measurement of the nerve conduction velocity (NCV) and electromyography (EMG) result almost always in abnormal values. The deep tendon reflexes are often depressed and a loss of sensation occurs frequently. A sural nerve biopsy is occasionally helpful to differentiate between different types of neuropathy, e.g. chronic inflammatory demyelinating polyneuropathy (CIDP) and CMT. The CMT phenotype overlaps sometimes with other diseases like DSS, Roussy-Levy-Syndrome or Friedreich Ataxia. In cases of doubt molecular genetic diagnosis can be helpful.

Genetic analysis

At this stage of the best practice guidelines only the CMT1A duplication and HNPP deletion is under consideration, further mutation analysis strategies will be included in a later version.

Since the vast majority of CMT1 patients carry a 1.5 Mb tandem duplication in chromosome 17p12 comprising the *PMP22* gene, the determination or exclusion of this mutation is the first step in a diagnostic procedure. Also the reciprocal deletion leading to HNPP is usually included in this first test. Since HNPP patients sometimes present with symptoms similar or identical to CMT type 1 it is important to include the deletion analysis in the first set of methods.

The HNPP test requires the same probes/markers and techniques as the screening of the CMT1A duplication. After exclusion or confirmation of a CMT1A duplication/HNPP deletion, further genetic analysis and/or clinical reexamination may follow.

The individual laboratories should state their limits or delineate what specific CMT testing is available, e.g. based on the method only duplication screening, also deletion screening and further mutation screening in the known genes.

In general two different methods are available:

Binary methods resulting in a "yes" or "no" answer.

- Pulsed field gel electrophoresis (PFGE) analysis of the CMT1A duplication/HNPP deletion.
- Fluorescence in situ hybridisation (FISH) analysis of the CMT1A duplication/HNPP deletion.

Dosage sensitive and other methods.

- RFLP Southern blot analysis (probes from the CMT1A/HNPP region).
- STR markers from the CMT1A/HNPP region.

- PCR methods based on the CMT1A-REP sequence to detect specific junction fragments.

Binary methods

In this category the detection of junction fragments by restricted whole genomic DNA and pulsed field gel electrophoresis (PFGE) as well as fluorescence in situ hybridization (FISH) methods are summarized (Tables 2 and 3). FISH can only be performed in expert and equipped labs. PFGE on a routine basis is widely used in the US, but rarely in Europe. The method leads to clear results, but has also limits, e.g. in the quality of blood samples obtained by the diagnostic center and the sophisticated labour intensive procedure required until a final autoradiogram is obtained. The failure rate (non-interpretable results) is estimated at about 10%.

FISH is based on Cosmids or other large insert clones (BACs, PACs) derived from the CMT1A region and containing *PMP22*, currently no commercial probe is available. This may change in the near future. To detect a 1.5 Mb duplication it is usually necessary to perform interphase FISH, and in order to determine the cell cycle state, use of a second probe from chromosome 17 is recommended. This ensures that a duplication is being observed and not a false positive signal due to replication during interphase. This results in a two colour approach. To obtain diagnostic security at least 50, better 100 nuclei, should be counted for the presence of 3 CMT1A signals relative to the second chromosome 17 probe. For the HNPP deletion this works also, but the deletion is on metaphase chromosomes clearly visible which reduces the necessary examination to about 10-30 metaphases. On a first glance this method looks ideal, but the quality and reliability of the FISH results depend strongly on the provided blood samples. Although FISH is in principle possible on different types of tissues the best results are obtained with heparinized blood and a short cultivation after arrival of the samples. The method has also a failure rate of up to 10-30% in some laboratories due to different reasons - using of wrong tubes by the external neurologists, e.g. tubes to obtain serum; long transportation and thereby decreasing quality of the prepared nuclei - but other laboratories have a greater than 99% success rate with this technology:

Dosage sensitive and other methods

The Southern hybridization methods which are widely used are shown in table 4. Especially the *MspI* blots are very common. Disadvantage of the *MspI* blot is a frequently observed homozygosity leading to non-informative RFLP patterns. Use of two probes subsequently (pVAW409R3a and pEW401HE) or simultaneously (pVAW412R3 and pEW401HE) helps



usually. It should be kept in mind that these probes are anonymous markers which may be rearranged without CMT1A duplication or HNPP deletion. The use of probe pNEA102 on EcoRI digested genomic DNA is not recommended for diagnostic purposes, the dosage differences for duplication/deletion are not strong enough (4->5 CMT1A, 4->3 HNPP). However, it is useful as additional probe and for scientific purposes. The pLR7.8 probe used on EcoRI/SacI digested DNA is very useful in cases where a junction fragment is detected. The dosage differences for recombinations outside the "hotspot" are hard to interpret and maybe therefore used only as indicative for a recombination which makes a second analysis necessary. As recent data show the CMT1A-REP elements are more polymorphic than expected, hence for accuracy also the finding of a junction fragment should be confirmed by another method. This holds also true if no change is detected which may simply indicate that the restriction site is deleted.

For STR based methods several protocols for multiplex PCRs are available (see table 5 for STR markers). It is not recommended to use only one marker, at least two of them should be positive for three alleles to be indicative for the CMT1A duplication. The HNPP deletion may be suspected when several polymorphic markers reveal only one allele (hemizygoty or homozygoty). In cases where only dosage differences are visible and not three alleles a second method should be used to define the type of mutation with diagnostic accuracy. The relative order of the RFLP/STR markers and CMT1A-REPs targeted PCR assays can be found in tables 6 and 7.

The PCR based methods for the CMT/HNPP junctions have been widely used in different laboratories. These applications showed two different things:

- The PCR is very difficult and hard to reproduce.
- The CMT1A-REPs are more polymorphic than expected which sometimes hampers the primer binding.

Hence these PCR methods are useful for scientific purposes, but not for a large scale diagnostic procedure. It should be kept in mind that only about 70% of the CMT1A duplication carriers show a junction fragment due to recombination within the hotspot region of CMT1A-REP.

Quantitative methods (table 8) are available and under development using the new generation of PCR machines which allow direct quantification during the amplification. However these methods are not very common and in cases of deeper interest the

corresponding laboratory should be contacted directly for further advice.

Materials

Usually DNA is extracted from peripheral blood lymphocytes and used for further analysis. For PFGE and FISH whole cells or nuclei are needed, also the establishment of lymphoblast cell lines is sometimes helpful. Different methods for DNA extraction are available, most common is a salting out method DNA, but also silica based extractions are reported.

Also other tissues depending on the method are suitable for analysis by DNA based methods or even FISH. However, epon embedded sural nerve biopsies are not useful.

Controls

In Southern hybridization experiments it is helpful to use DNA with a typical CMT1A duplication, HNPP deletion and a healthy control tested with different methods, e.g. MspI blot, EcoRI/SacI blot, FISH, polymorphic markers for control purposes in every blot. This helps to identify e.g. partial digestions, but also polymorphisms in the RFLPs. For PCR based method the same holds true.

Prenatal diagnosis

A prenatal diagnosis is offered by some of the centers for CMT. Since prenatal diagnosis as well as preimplantation diagnosis is regulated by national laws and the inquiries are rare it is recommended that the individual laboratories/countries find their own rules until European laws are available. Anyway a prenatal diagnosis has some general features different from an individual diagnosis for a single patient. First, in cases of CMT the mutation of one of the parents should be known. Second, as usual, in case of an affected mother a maternal contamination of the analysed amniotic cells/chorionic villi has to be excluded by microsatellites. In case of a paternal inherited disease it should be determined also by microsatellites that really embryonic tissue has been analysed.

Linkage analysis

Linkage analysis may be still helpful in cases with a clear phenotype but no detectable mutation, e.g. if no duplication is found but a mutation in *PMP22* is assumed but not yet detectable (e.g. intronic mutations).

Probes

All probes mentioned in these guidelines are distributed by the European CMT Consortium and the national contact laboratories, bacterial strains carrying the corresponding plasmids can be grown in every



laboratory performing the diagnostic procedures. Commercially available probes approved for diagnostic purposes are currently not known.

PCR primers

Primer sequences, PCR conditions etc. are on several homepages public available (see point 12.).

Interpretation

The detection of a CMT1A duplication or HNPP deletion can be interpreted as disease causing mutation for the neuropathy patients. However, it may be necessary to repeat the clinical analysis because not all of the duplication carriers show a NCV below the cut off value of 38 m/s, and also deletion carriers may present with a more CMT like phenotype. Rarely, patients diagnosed as Friedreich Ataxia turn out to be CMT1A duplication carriers. In case where no mutation can be found and the clinical diagnosis is free of doubt a further mutation screening is recommended.

Web resources

- <http://www.neuro.wustl.edu/neuromuscular/nother/myelin.html>
- <http://imgen.bcm.tmc.edu/molgen/lupski/>
- <http://www.almc.com/~smith/ResearchOrg.html>
- <http://www.mdausa.org/>
- <http://molgen-www.uia.ac.be/CMT/>
- <http://www.cmtint.org>
- <http://www.charcot-marie-tooth.org>
- <http://www.ncbi.nlm.nih.gov/disease/Charcot.html>
- <http://www.muscular-dystrophy.org>

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Table 5

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- Haupt et al. (1997) Hum. Genet. 99:688-691
- Yamamoto et al. (1998) Hum. Mut. 11:109-113
- Chang et al. (1998) Clin. Chem., 44:270-274
- GenBank n° 41165 and 41166
- Stronach et al. (1999) J. Per. Nerv. Syst., 4:117-122
- Bernard et al. (2000) Eur. J. Hum. Genet., 8:229-235

Table 7

- <http://www.bcm.tmc.edu/molgen/lupski>
- GenBank n° U41165 (distal) and U41166 (proximal)

Table 8

- Young et al.(1998) Neurology 50:760-76

Table 1. Nomenclature and gene ID

OMIM	OMIM Name	Gene Name	Inheritance	GDB ID
#118220 *601097	CMT1A	Peripheral myelin protein 22 (<i>PMP22</i>)	AD	134190
#118200 *159440	CMT1B	Myelin Protein Zero (<i>MPZ, PO</i>)	AD	125266
601098	CMT1?	Early Growth response gene 2 (<i>EGR2</i>) ?	AD	120611
#145900 *601097 *159440	Dejerine Sottas Syndrome (DSS)	<i>PMP22/MPZ/EGR2</i>	AD/AR	
#605253 *159440.0013	Neuropathy, congenital hypomyelinating	<i>EGR2/MPZ</i>	AR	120611/125266
*214400	CMT4A	?	AR	
*601382 *603557	CMT4B	<i>MTMR2</i>	AR	9957577
	CMT4C	?	AR	
*601596	CMT4D	?	AR	
*601455	CMT4F (Lom)	N-myc downstream-regulated gene 1 (<i>NDRG1</i>)	AR	9958844
#302800 *304040	CMTX	Connexin32 (<i>Cx32, GJB1</i>)	XD	125246
*302801	CMTX2	?	XR	
*302802	CMTX3	?	XR	
#162500 *601097	HNPP	<i>PMP22</i>	AD	
*118210	CMT2A	?	AD	
*600882	CMT2B	?	AD	
*158580	CMT2C	?	AD	
*601472	CMT2D	?	AD	
	CMT2E	Neurofilament light gene (<i>NEFL</i>)	AD	168635
	ARCMT2A	?	AR	



Table 2 PFGE overview

Locus	Clone	Subclone	Cloning site	Insert size	Method	Junction fragment
D17S122	pVAW409R3	pVAW409R3a	EcoRI-BamHI	1400 bp	SacII PFGE	500 kb CMT1A junction
					FspI PFGE	500 kb CMT1A junction
					AscI PFGE	500 kb CMT1A junction
CMT1A-REP	c20G2	pNEA102	EcoRI	1800 bp	SacII PFGE	500 kb CMT1A junction 820 + 770 kb HNPP junction
					EagI PFGE	150 kb CMT1A junction 320 (370) kb HNPP junction
CMT1A-REP	c56A5	cosH1	EcoRI	?	EagI PFGE	150 kb CMT1A junction 300 (350) kb HNPP junction

Table 3 FISH overview

Locus	Clone	Labelling	Method
proximal CMT1A-REP	c74F4	FITC avidin	FISH
distal CMT1A-REP	c112C10	FITC avidin	FISH
CMT1A monomer unit	pVAW409R1	FITC avidin	interphase FISH
<i>PMP22</i>	c103B11, c132G8	digoxigenin	interphase FISH
<i>PMP22</i>	c77F4, c132G8	digoxigenin	interphase FISH

Table 5 STR analysis

Locus	STR	Type of STR	Allele sizes (pb)	Number of alleles	PIC	H
D17S122	RM11-GT	CA-repeat	153-167	8		74
D17S261	Mfd41	CA-repeat	157-171	6	0,44	
D17S921	AFM192xh12	CA-repeat	174-183	10	0,69	0,73
D17S1356	142E8ac1	CA-repeat	145-157	7		0,48
D17S793	AFM165zd4	CA-repeat	99-109	7		0,7
D17S839	AFM200yb12	CA-repeat	155-175	5	0,47	0,56
D17S955	AFM317yg1	CA-repeat	187-181	4	0,4	0,45
D17S1357	103B11ac1	CA-repeat	194-210	6		0,57
D17S1358	133C4ac1	CA-repeat	182-194	7		0,74



Table 6 Relative order of the RFLP/STR markers according to K. Inoue & JR Lupski, unpublished observations.

Proximal CMT1A-REP
 D17S261
 D17S122
 D17S1357
 D17S1356
 D17S125
 D17S839
 D17S1358
 D17S61
 D17S955
 D17S921
 Distal CMT1A-REP

Table 7 CMT1A-REPs targeted PCR assays.

Locus	Method	Primer name
CMT1A-REP sequence proximal and distal	DNA sequencing of 6 controls	none
CMT1A-REP	PCR analysis of junction fragments + EcoRI and NsiI digestions	distF proxR proxF distR
CMT1A-REP	PCR analysis of junction fragments + NsiI digestion	DF1 (pos 1781-1805, distal CMT1A-REP) DF2 (pos 2394 - 2418, distal CMT1A-REP) DR1 (pos 5077 - 5101, distal CMT1A-REP) DR2 (pos 3574 - 3598, distal CMT1A-REP) PR1 (pos 5069 - 5093, proximal CMT1A-REP) PR2 (pos 3560 - 3584, proximal CMT1A-REP)
CMT1A-REP	PCR analysis of junction fragments + NsiI and AccI digestion	primer A (pos 1785 - 1806, distal CMT1A-REP) primer B (pos 5069 - 5093, proximal CMT1A-REP) primer C (pos 3751 - 3771, distal CMT1A-REP) primer D (pos 3489 - 3509, distal CMT1A-REP)
CMT1A-REP	PCR analysis of junction fragments + EcoRI digestion	CMT1A-FOR and HNPP-FOR CMT1A-REV HNPP-REV
CMT1A-REP	PCR analysis of junction fragments + EcoRI and NsiI digestions + EcoRI and SacI digestions	Rdist1 (pos 1500-1523 of sequence HSU41165) Rprox2 (pos 5177 - 5154 of sequence HSU41166)



Table 8 Quantitative PCR

Fragment	Primer and Label
PMP22 exon 4	None (reverse) FITC (forward)
CETP (reporter fragment)	None (reverse) FITC (forward)



Table 4 RFLP Southern blot methods for dosage differences and junction fragments.

Locus	Clone	Subclone	Cloning site	Insert size	Polymorphism	Allele sizes	Allele frequencies
D17S122	pVAW409R1	pVAW409R1b	EcoRI-BamHI	2500 bp	MspI RFLP	5.3 kb 2.7 + 2.6 kb	0,86 0,14
D17S122	pVAW409R3	pVAW409R3a	EcoRI-BamHI	1400 bp	MspI RFLP	2.8 kb 2.7 kb 1.9 kb	0,5 0,44 0,06
D17S125	pVAW412R3	pVAW412R3HEb	EcoRI-HindIII	1300 bp	MspI RFLP	10.5 kb 5.4 kb	0,17 0,83
D17S125	pVAW412R3	pVAW412R3HEc	EcoRI-HindIII	800 bp	MspI RFLP	2.6 kb 0.7 + 1.9 kb	0,83 0,17
D17S61	pEW401	pEW401HE	EcoRI-HindIII	850 bp	MspI RFLP	5.5 kb 4.4 kb 4.7 kb	0,24 0,76 rare
<i>PMP22</i> (cDNA)	rat human	pCD25F3 p132-G8R1	FspI-EcoRI	10 kb	EcoRI/HincII RFLP EcoRI/HincII RFLP	11 kb 9.6 kb	dosage
CMT1A-REP		pNEA101	EcoRI				dosage
CMT1A-REP	c20G2	pNEA102	EcoRI	1.8 kb	EcoRI	7.8 + 6.0 kb dosage	dosage
CMT1A-REP	pLR6.0	pLR6.0	EcoRI	6 kb	EcoRI/SacI/NsiI EcoRI/SacI/NsiI	7.8 kb 1.7 kb	HNPP junction CMT1A junction dosage
CMT1A-REP	pLR7.8	pLR7.8	EcoRI	7.8 kb	EcoRI/SacI EcoRI/SacI	3.2 kb 7.8 kb	CMT1A junction HNPP junction dosage
CMT1A-REP	proximal	pHK1.0P	EcoRI/PstI	1 kb	EcoRI	2.3 + 3.2 kb	CMT1A dosage
CMT1A-REP	proximal	pHK5.2P			EcoRI/HindIII	3.2 + 3.4 kb	CMT1A dosage
CMT1A-REP	proximal	pJ7.8P	EcoRI	7.8 kb	EcoRI/SacI	3.2 kb 7.8 kb	CMT1A junction HNPP junction dosage
	proximal	pJ5P	EcoRI	5 kb	EcoRI/HindIII	3.3, 3.1, 1.8, 1.7	dosage