

MEETING REPORT

EMQN Best Practice guidelines for diagnostic testing of mutations causing non-syndromic hearing impairment at the DFNB1 locus

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INTRODUCTION

Clinical background

Hearing impairment is the most common sensory disorder, affecting one in every 500–1000 newborns (<http://hearing.screening.nhs.uk/nationalprog>). It is estimated that about half of these have a genetic cause, whereas the other half are caused by environmental factors, such as rubella or CMV infection during pregnancy, factors associated with prematurity or ototoxic medication. In most genetic cases, the inheritance pattern is autosomal recessive (80%), but also autosomal dominant (17%), X-linked (2–3%) and mitochondrial (<1%) inheritance has been described. In about 30% of cases, additional clinical and/or physical features lead to a syndrome diagnosis, but in the remaining 70% the only finding is hearing impairment in otherwise healthy people. Non-syndromic hearing loss is genetically very heterogeneous, with over 150 associated loci and >60 identified causative genes (<http://hereditaryhearingloss.org/>). Remarkably, defects in one locus (*DFNB1*) account for up to 50% of cases in many populations, which makes this the most common cause of non-syndromic, prelingual hearing impairment and deafness. The locus was described for the first time in 1994¹ and the first mutations in 1997.^{2,3} The *DFNB1* locus (OMIM^{*} 220290) contains two genes associated with hearing loss, *GJB2* (OMIM^{*}121011) and *GJB6* (OMIM^{*}604418), encoding connexin 26 (CX26) and connexin 30 (CX30), respectively. The reference sequences are given in Table 1. Connexins aggregate to form connexons, consisting of six connexin proteins in heteromeric or homomeric complexes located at gap junctions, and which are involved in the transport of ions and other low-molecular weight components between cells.

The severity of hearing impairment is graded as mild (20–40 dB), moderate (41–55 dB), moderately severe (56–70 dB), severe (71–95 dB), or profound (>95 dB) and may involve low (<500 Hz), middle (500–2000 Hz), or high (>2000 Hz) frequencies. Non-syndromic hearing impairment caused by mutations in the genes at the *DFNB1* locus is associated with congenital, generally non-progressive, sensorineural hearing impairment that is moderate to profound by pure tone audiometry, or auditory brain stem response testing (ABR). It tends to affect all frequencies. The vast majority of cases therefore fail Newborn Hearing Screening, but a few rare cases of later-onset hearing impairment^{4,5} and some progression of the

hearing loss have been described.^{6–8} By definition, there are no related systemic findings identified on medical history and physical examination. Family history is consistent with autosomal recessive inheritance, but pseudodominant inheritance should not be overlooked, especially in marriages where both partners have hearing loss (deaf–deaf unions).⁹

Genetic background

The most common mutation in *GJB2* associated with deafness varies around the world according to population. In Caucasians, the c.35delG is the most frequent mutation, comprising 70% of alleles in some populations, with a carrier rate of 1–3% in the general population.^{7,10–14} The c.167delT mutation is the most common mutation in the Ashkenazi Jewish population,^{15,16} c.235delC is found more frequently in the Japanese populations,¹⁷ and p.Trp24* in the Indian, Bangladeshi, Romany, and Slovak populations as well as others.^{18–22} The splice mutation c.−23+1G>A is found in several populations (including Caucasian, Bangladeshi, Egyptian, Algerian, Czech, Turkish, Mongolian, and Chinese).^{18,23–28} Snoeckx *et al*²⁹ have compiled data from many centres in an effort to try to establish correlation between genotype and phenotype. They were able to distinguish between ‘severe’ alleles, which tended to be truncating, and ‘mild’ alleles, often non-truncating missense or splice mutations, but many could not be graded due to their relatively rare occurrence in the population.

Although many mutations have been described within the *GJB2* gene, it became clear that there must be additional alleles. Several investigators described a common, partial deletion (342 kb, del(*GJB6*:D13S1830) in the *GJB6* gene, which seemed to account for many of the missing alleles.^{30–32} Later, it was confirmed that this is in fact a 309-kb deletion.³³ Rarer deletions have subsequently been identified, all involving a minimally deleted region between *GJB6* and *CRYL1*^{34–36} (Figure 1).

It should be noted that a few mutations in the *GJB2* gene have been associated with autosomal dominant inheritance (DFNA3),^{37–39} which may be non-syndromic⁴⁰ or with associated skin features^{41–48} in syndromic forms of deafness such as Vohwinkel syndrome, Keratitis Ichthyosis Deafness, Bart Pumphrey syndrome, Palmo Plantar Keratoderma, and Hystrix-ichthyosis. These are particular mutations involving specific residues of the protein.

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Indications for testing and information needed

Any person with bilateral non-syndromic sensorineural hearing impairment of prelingual onset with no known aetiology can be offered testing. Cascade testing (successive testing of relatives to follow the mutations) can be offered where an index case has been identified, with one or two mutations in the genes at the *DFNB1* locus. In addition, partners of index cases as well as partners of known carriers can also be offered testing. Prenatal diagnosis can be offered where both parents are identified as carriers of definitely pathogenic mutations. In our experience, this request is extremely rare and is not a common practice in many countries.

It is useful to include the following details with a request for testing: family history and pedigree, imaging results (for example CT or MRI scan results), audiograms (or ABR for small children), ethnicity, and consanguinity.

MATERIALS AND METHODS

This article is the output from a Best Practice meeting on deafness caused by mutations at the *DFNB1* locus, organised by the European Molecular Genetics Quality Network (EMQN; <http://www.emqn.org.uk>). The EMQN is an independent organisation based in the United Kingdom that promotes improvement in the quality of genetic testing by ensuring that diagnostic molecular genetic laboratory test results are accurate, reliable, and comparable wherever they are produced. The mechanism for doing this is the organisation of external quality assessment (EQA) schemes and the development of Best Practice guidelines. Annual participation in an EQA scheme is necessary for quality assurance, continuous validation, evaluation of reporting, and continuous education of laboratory staff. Testing should only be performed in laboratories that are accredited according to ISO15189 or the equivalent (see the Organisation for Economic Cooperation and Development (OECD) Guidelines (<http://www.oecd.org/science/biotechnology/policies/38839788.pdf>)).

Clinicians and Scientists involved in molecular diagnosis of deafness were invited to a workshop supported by EMQN in Nijmegen, Netherlands in October 2009 to discuss and formulate consensus-based Best Practice Guidelines for the molecular diagnosis of deafness caused by mutations at the *DFNB1* locus. The meeting was advertised on the EMQN and EuroGentest websites (<http://www.eurogentest.org>); it was organised by the EMQN team responsible for the *DFNB1* EQA scheme and attended by 15 clinical and scientific experts named in the Acknowledgements. Discussions were based

Table 1 Accession numbers for the *GJB2* and the *GJB6* genes

	GenBank accession	Swiss-Prot
<i>GJB2</i> (exons 1 and 2)	NM_004004.5	CX26: P29033.3
<i>GJB6</i> (exons 3, 5, and 6)	NM_006783.4	CX30: O95452

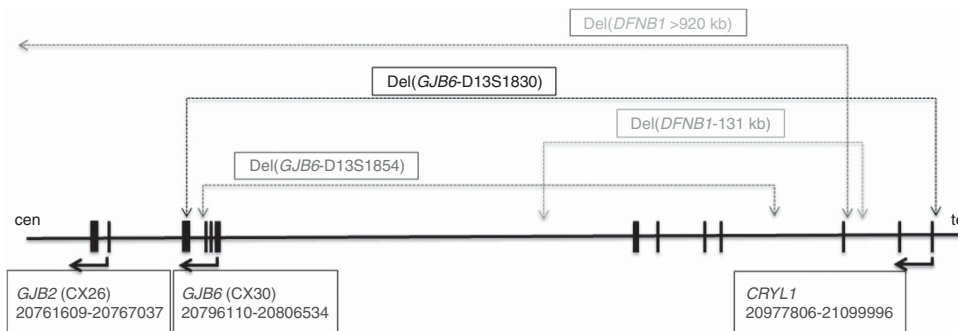


Figure 1 Summary of the deletions identified at the *DFNB1* locus. Adapted from Wilch *et al.*³⁴ g. numbering is according to NCBI built 37 (GRCh37) del(GJB6-D13S1830) = chr13: g.20797177_21105945del ~ 309 kb³⁰⁻³² del(GJB6-D13S1854) = chr13: g.20802727_21034768del ~ 232 kb³⁶ del(DFNB1-131 kb) = chr13: g.20939343_21070698del ~ 131 kb³⁴ del(DFNB1 > 920 kb) = chr13: g.(20132969-20143922)_(21063547-21065406)del > 920 kb³⁵.

upon a pilot EQA scheme for *DFNB1* testing held in 2008–2009 by EMQN. After the meeting, draft guidelines were circulated for comment to the attendees and subjected to further revision.

RESULTS AND DISCUSSION

Molecular diagnostic testing at the *DFNB1* locus/approaches and protocols

Laboratory analysis. Analysis of c.35delG alone, or specific combinations of point mutations, is no longer considered sufficient for diagnostic testing. In some laboratories, it may be desirable to screen for specific prevalent mutations first, but in the absence of biallelic mutations it is recommended that all possible mutations should be screened, for adequate diagnostic testing. This entails sequence analysis of the coding sequence (exon 2) and analysis of the splice sites (5' and 3' ends of intron 1), as well as detection of common deletions in the *GJB6* gene associated with recessive non-syndromic hearing loss. The multiplex PCR assay designed by del Castillo *et al.*³⁶ is used to detect the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854). These deletions are relatively common in some populations,^{30,49-52} but appear to be very rare in others⁵³⁻⁵⁷ and should be sought at least in those in whom only a single mutation has been found on *GJB2* screening. GeneClinics advises that very few individuals (<0.5%) will be homozygous for two *GJB6* deletion alleles;⁵⁸ however, as the assay is straightforward, consanguinity not uncommon and ethnic origin not always stated, we suggest that this is done in all cases. The MLPA kit made by MRC Holland, Amsterdam, Netherlands, (P163-D1 GJB-WFS1) allows the detection of large *GJB6* deletions, but as it lacks the specific probes for del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) it cannot detect them explicitly; although there are added probes for specific point mutations such as c.35delG, c.-23 + 1G>A, c.167delT, c.235delC, and c.313_326del, these are insufficient for comprehensive diagnostic testing in our opinion. In addition, the P163-D1 kit includes probes for other genes that are not associated with deafness caused by mutations in the genes at the *DFNB1* locus, including progressive syndromic forms of deafness, which has the inherent risk of overtesting.

As point mutations in the *GJB6* gene have been associated with syndromic deafness, including skin abnormalities, rather than non-syndromic forms of deafness, sequencing of this gene is not recommended in *DFNB1* analysis and is also considered to be overtesting. See Box 1 for an overview.

Reporting. Reports should contain concise background information, an outline of the techniques and strategy used for screening, and

Box 1 Standards for diagnostic testing and reporting of mutations at the *DFNB1* locus

Screening	Direct sequencing of coding exon 2 Analysis of splice sites Exclusion of common deletions encompassing <i>GJB6</i> , confirmed by multiplex PCR if MLPA is first screened
Reporting	Report should ideally be no more than one page Brief description of methods used Expected sensitivity of the methods, particularly, where one or no mutations are found. Reference sequence and version used to denote numbering of nucleotides Mutation given at nucleotide level with predicted protein effect Use of HGVS nomenclature for description of variants/mutations (See suggestions for common deletions) Brief interpretation of findings and further recommendations

Abbreviation: HGVS, the Human Genome Variation Society.

prominent display of results together with interpretation of the findings. Although most laboratories write reports to the referring clinical geneticists, it is very likely that in future, reports may be sent directly to general physicians and paediatricians. It is suggested that ideally reports should be on a single page. Guidelines already exist, for example, those of the OECD (<http://www.oecd.org/science/biotechnology/policies/38839788.pdf>), the Swiss Society of Medical Genetics (<http://www.smgm.ch>), and the UK Clinical Molecular Genetics Society (www.cmgs.org); however, the following relates specifically to deafness caused by mutations at the *DFNB1* locus. See Box 1 for an overview.

(i) Nomenclature. Mutations should be annotated according to the Human Genome Variation Society (HGVS) guidelines. Although numerous publications over the years have reported mutations using different nomenclatures, it is important to name the 'known' mutations following HGVS. Furthermore, it is necessary to indicate the reference sequence and version number used for annotation. (<http://www.hgvs.org/mutnomen/>). Mutation nomenclature can be checked using programmes such as Mutalyzer and Alamut. For example, 30delG should be named c.35delG, 310del14 (also known as 312del14, 314del14) should be named c.313_326del, and -3172G>A (also known as -3172 or IVS1+1G>A) should be c.-23+1G>A. It is still acceptable to refer to old nomenclature as long as HGVS is also indicated. In addition, any new variant should be annotated according to HGVS guidelines. For reporting purposes, it is recommended to use annotation at *both* the nucleotide and the protein level, as different nucleotide changes can lead to the same change at the protein level due to the degenerate code at the third nucleotide of a codon.

Once mutations have been found, testing of parents is recommended to establish phase and recurrence risks (there is the possibility of *de novo* mutation, large deletion, uniparental disomy, or allelic dropout). When genotypes are reported, phase should be considered, for example, c.[35delG];[139G>T] indicates that the mutations are known to be on different alleles, whereas c.[35delG(;)139G>T] indicates that phase is unknown, and c.[35delG];[(35delG)] denotes a homozygous 35delG, not confirmed by analysis of both parents.

There is considerable difficulty, however, in using HGVS for the well-known deletions including those found in the *GJB6* gene (<http://www.hgvs.org/mutnomen/uncertain.html#exondel>). We suggest that for reporting purposes, it is acceptable to use original deletion names that is, del(*GJB6*-D13S1830). The combination of a mutation in *GJB2* and a deletion in *GJB6* should be described as a compound heterozygote, instead of a double heterozygote, because the deletions likely involve a regulatory element of *GJB2*. Therefore, a common genotype might be described as [GJB2:c.35delG];[GJB6:del(*GJB6*-D13S1830)].

When an MLPA kit has been used to identify deletions, it is not possible to be sure of the exact breakpoint of the deletions detected, and therefore, we suggest that for reporting purposes deletions detected using MLPA should be validated using the multiplex PCR method described by del Castillo *et al.*³⁶

(ii) Mutations and variants of unknown clinical significance. Alterations at the *DFNB1* locus can be classified as truncating and non-truncating. New variants, if predicted to lead to premature stop codon, frameshift, or splicing alteration with abolition of the obligatory AG/GT sites, all presumed to interfere with proper protein production, should be labelled as pathogenic or truncating alterations.

More difficult are the novel missense or synonymous changes. In laboratory reports, an interpretation should be made as to the predicted functional effect of the variant, following the guidelines of the Clinical Molecular Genetics Society (<http://cmgs.org/>). See also Table 1.

(iii) Controversial known variants. A few variants are notoriously difficult to interpret, the most well-known example being the c.101T>C p.(Met34Thr) variant. This variant has been labelled as autosomal dominant, autosomal recessive, and a neutral variant, but current evidence suggests it is most likely to be a hypomorphic recessive allele^{3,29,59,60} (not fully inactivating the protein). Indeed, Snoeckx *et al.*²⁹ have shown that 38 patients who were genotyped c.[35delG];[c.101T>C] had mild to moderate hearing loss. If the mutation had no specific influence on the phenotype, this would have resulted in a variety of degrees of hearing loss. Moreover, the high prevalence of this variant in the general Caucasian population (carrier rate of 1/37–1/43)^{11,14,52} and its under-representation in several cohorts with profound deafness, supports its role as a hypomorphic allele rather than a mutation responsible for severe to profound hearing loss. Therefore, if p.(Met34Thr) is present in homozygosity, or with another pathogenic mutation in a patient with mild hearing loss, it is likely to be causative; if the hearing loss is profound however, another cause should be considered.

For p.(Val37Ile), evidence now suggests that it is a common mutation causing mild–moderate hearing loss, especially, in subjects from Southeast Asia and North Africa⁶¹; the variant should be reported as such. The variants p.(Arg127His), p.(Val27Ile), p.(Lys224Gln), and p.(Val153Ile) appear to be non-pathogenic polymorphic variants.

(iv) Interpretation. This is the final step in reporting and is the professional role of the diagnostic laboratory. For diagnostic testing, the common situations include:

(1) Two mutations found (both pathogenic, or one pathogenic and one unknown); the results confirm (if mutations are shown to be *in trans*) or support the diagnosis (if phase is unknown). It is good practice to mention the effect of some of the milder mutations (see above, and Table 2). The report should recommend clinical genetic counselling when one or two mutations are detected. Carrier screening can be offered to other family members.

Table 2 List of the main pathogenic missense variants and associated phenotypes (according to Snoeckx *et al*²⁹)

cDNA mutation	Protein	Clinical phenotype
c.1A>G	p.(Met1Val) (p.(Met1?))	
c.95G>A	p.(Arg32His)	
c.101T>C	p.(Met34Thr)	Mild
c.109G>A	p.(Val37Ile)	Mild
c.229T>C	p.(Trp77Arg)	Profound
c.246C>G	p.(Ile82Met)	
c.269T>C	p.(Leu90P)	Mild-moderate
c.283G>A	p.(Val95Met)	Profound
c.427C>T	p.(Arg143Trp)	Profound
c.551G>C	p.(Arg184Pro)	Severe
c.617A>G	p.(Asn206Ser)	Mild

- (2) No mutation is found; hearing impairment is genetically very heterogeneous, therefore, this result does not exclude other genetic or acquired causes of hearing impairment in this patient. The sensitivity of the test strategy should be given; sequencing *GJB2* (noncoding and coding exons) and screening for del(*GJB6-D13S1830*) gives >98% mutation detection rate. For parents of a deaf child who does not have mutations at *DFNB1*, residual recurrence risk should be estimated. This is likely to be a minimum of 10% but up to 25% (as for any recessive disorder).
- (3) One mutation found; the patient is a carrier, which supports the diagnosis, but for mutations with high frequencies this can be a coincidental finding. In familial cases (siblings) where one mutation is found, haplotype analysis (marker analysis) may be recommended. If haplotype analysis shows all affected siblings to have inherited the same alleles from their parents, the diagnosis of hearing loss caused by a gene at the *DFNB1* locus is supported, but if not, carrier status is likely to be coincidental to the aetiology of hearing loss. Suggested markers for this analysis are D13S141-(*GJB2-GJB6*)-D13S175-D13S143-D13S1236, although other markers could be useful as well.
- (4) New missense variation: audiometry of the parents may be recommended when they carry the variant in order to exclude dominant inheritance. In contrast, if a single new missense variant is shown to have arisen *de novo*, then it is possible that it may be a pathogenic autosomal dominant mutation.

Carrier testing is usually performed in the case of cascade testing (successive testing of family members to follow the mutation), and genetic counselling is part of the recommendation if the subject is found to have a mutation. Partners of carriers should be offered testing as well, because of the high incidence of carriers in the general population.

CONCLUSIONS

Diagnostic testing for *DFNB1*-associated hearing impairment is technically simple compared with many diagnostic tests and has a good diagnostic yield (up to 15–20% of singleton cases and 50% of recessively inherited cases of deafness). We have made recommendations for good practice for diagnostic testing of this locus, as well as reporting of the results, which includes sequence analysis of the coding region of the *GJB2* gene, as well as of the splice sites and a search for common deletions involving *GJB6*. Nomenclature of mutations should follow HGVS guidelines, and mutations should be annotated at the protein and cDNA level. It is the role of the

diagnostic laboratory to provide an interpretation of the results when reporting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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