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Diagnostic Testing for Prader-Willi and Angelman Syndromes: Response

To the Editor:

The letter by Monaghan et al. (1997) in the January 1997 issue of the *Journal,* discussing testing for Prader-Willi (PWS) and Angelman (AS) syndromes, raised several interesting points. Essentially the issues related to the sequence of diagnostic testing for PWS/AS (cytogenetics, methylation testing, and FISH), considering the pick-up rate of abnormal results and the costs involved. Several others have addressed this topic (Chu et al. 1994; Smith et al. 1995; Young 1995; American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996; Erdel et al. 1996; Kubota et al. 1996), but Monaghan et al. (1997) are the first to include discussion of the costs of testing. We would like to (*a*) relate our 12-mo experience of performing diagnostic tests for a referred group of patients suspected to have PWS or AS and (*b*) review the logistics and costs of multiple testing.

Our department of cytogenetics is well established, with a specimen receptionist, an excellent blood laboratory, and staff dedicated to FISH. Our newly instituted molecular laboratory has one staff scientist who performs DNA extractions and the methylation test. A staff cytogeneticist (A.S.) consults with patients/referring clinicians, obtains clinical information, and coordinates the whole process. Within the one department, the whole is well accommodated, with adjacent laboratories and offices. All concerned work closely together.

Cytogenetic analysis on high-resolution chromosomes (HRC) had been performed for many years in our laboratory. FISH for PWS/AS was piloted in 1991 and replaced HRC in 1993. We began methylation analysis in 1995. Our aim was to obtain a result on the index case from a single blood collection, using a coordinated approach. Routine cytogenetics and DNA extraction would be performed simultaneously. The suspension from the cytogenetic harvest would be forwarded to the FISH laboratory. Methylation analysis would be performed on an aliquot of the extracted DNA. Finally,

FISH would be performed only on those cases with an abnormal methylation pattern. This seemed a logical and cost-effective testing protocol.

Blood samples received in the laboratory were accompanied by a referral form. The requested amount was 10 ml, but frequently samples from babies were only 1–3 ml, those from older children 5 ml. The blood sample was split on arrival in the laboratory. An aliquot of 0.5 ml was retained for routine 72-h cytogenetic culture, followed by harvest and GTG banding. The remainder was given to the molecular laboratory, and DNA was extracted by a simple salting-out technique (Miller et al. 1988) and was stored. The suspension remaining after the cytogenetic harvest was sent to the FISH laboratory and was stored at −20°C. A cytogenetic result was obtained, usually within 15 d, and, if this was normal, the methylation test was perfomed on an aliquot of the extracted DNA. For methylation analysis, enzymes *Hin*dIII and *Hpa*II were used with P^{32}-labeled probe PW71B (kindly donated by B. Horsthemke, Essen), by standard Southern blotting techniques (Dittrich et al. 1992). If the methylation pattern diagnosed PWS or AS, then FISH was performed on slides redropped from the stored suspension, by use of probe SNRPN or probe D15S10 (Oncor or Vysis). For cases not deleted by FISH, parents' bloods were requested and DNA was extracted. CA repeats for uniparental disomy were then performed on all three specimens.

During the first 12 mo, 204 patients were referred, 71 for PWS and 133 for AS. Results are shown in table 1, together with a broad clinical evaluation. Both PWS and AS were overdiagnosed, perhaps because of clinician enthusiasm to further test unresolved cases. The methylation test gave a characteristic abnormal result in all classical PWS patients—an additional three patients with abnormal methylation require further clinical evaluation. AS was more problematic, as would be expected on the basis of the more complex genetic etiology. Only 51% of classical AS patients showed an abnormal methylation pattern—the expectation was 75%–80% (American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996)—and an additional four patients need to be reassessed. FISH was not performed in 54 cases, representing a substantial saving among the 152 cases of nonclassical phenotype. On the other hand, FISH was performed first in 25 (35%) of the PWS patients and in 73 (55%) of the AS patients, for whom a normal methylation pattern was later found. This "early" FISH testing, welcomed by the referring doctor, was unnecessary, offsetting the benefit of the logical approach.

Table 1

Results of Diagnostic Testing for Patients Suspected to Have PWS or AS

	Total No. of Patients with Syndrome/No. (% of Total) of Patients with Classical Phenotype[a]	
	PWS	AS
Referrals	71/17 (24)	133/35 (26)
Methylation-pattern status:		
Normal	51/0	111/17
Abnormal	20/17	22/18
FISH status:		
Performed	55/. . . (77)	95/. . . (71)
Not performed	16/. . . (23)	38/. . . (29)

[a] Diagnostic criteria for the PWS classical phenotype are from Holm et al. (1993); consensus diagnostic criteria for AS are from Williams et al. (1995).

Logistics of Multiple Testing

In reviewing our results, we find that several points are worth highlighting. With respect to methylation analysis, a number of problems were encountered in establishing the laboratory; the blood specimens received for DNA extraction were frequently inadequate (particularly in babies) once the requirements of cytogenetics had been met; the radioactive P^{32} was obtained fortnightly; the methylation tests were batched (once every 3–4 wk), because of the variable numbers received each week; occasionally the blood needed to be frozen and DNA was extracted later. Thus there were delays in the methylation results, with turnaround time for a result being 1–3 mo, depending on the need for a repeat test, availability of fresh P^{32}, and staff availability. As many as 50% of the methylation tests were repeated, but one would expect this percentage to improve. On the other hand, the cytogenetic/FISH laboratory receives many requests for FISH for a range of microdeletion syndromes, so that these are processed at least once per week and there is no back log. The FISH result (routinely on 50 cells) was available in 2–3 d, and overall only 5% of FISH tests needed to be repeated to obtain optimal quality.

Coordinating the progress of specimens was the greatest task, even in our "ideal" setting. Considerable time was spent in data entry, updating the progress of specimens, obtaining clinical information, storage of suspensions, organizing blood recollection because of unsatisfactory DNA specimens, answering phone calls, and educating clinicians about the testing protocol. The component of the coordination of multiple tests cannot be ignored. In reality, we did not adhere to our desired protocol very stringently—in many cases FISH was the only test performed, and, in 95% of the cases in which both tests were performed, the FISH test result was actu-

ally available first. The logistics of how multiple testing can best be coordinated has not been discussed before.

The Costing of Tests

The most significant factor in the costing of all tests is the salary of the scientists performing the tests, plus any supervisor/coordinator's salary. These salary costs are usually dependent on personnel gradings, and laboratories use staff at different academic/grading levels, so they may not be directly comparable. The next major cost is that of the probes. Commercially available probes need to be purchased on a regular basis and involve a substantial capital outlay. Probes grown in-house are frequently considered to be "free," but a significant staff component is required in the growing, purifying, and labeling of these probes. A detailed cost analysis may show the differences to be neglible. A further important factor is the cost of laboratory overheads (e.g., water, electricity, land rates, maintenance, etc.), which, in some cases, may not be directly billed to the laboratory.

In the letter by Monaghan et al. (1997), a cost of US$200.00 was given for a FISH test and a cost of US$300.00 was given for a methylation test. Details of how these costs were derived were not provided (nor was it specified whether they represent either the amount billed to the patient or actual costs), so it is not possible to make valid comparisons with the costs incurred by these tests in other laboratories.

The actual cost of FISH in our laboratory is AU$288.00/test (the exchange rate on 3 April 1997 was AU$1.00 to US$0.7957). This comprises an average AU$38.00 for reagents (depending on probe) and AU$250.00 for staff. This excludes overhead costs, borne by the hospital. To obtain an idea of the cost of a FISH test overall in Australia, a survey, commissioned by the Australasian Society of Cytogeneticists, arrived at a minimal cost of AU$250.00/test, if overhead laboratory maintenance was not included, or AU$500.00, if these costs were included.

In our laboratory, the actual cost of methylation testing, including DNA extraction, with donated probe, is AU$276.00/test (comprising an average of AU$26.00 for reagents, plus staffing and overhead factors similar to those for FISH). An informal meeting, in association with the International Prader-Willi Syndrome Organisation Conference in Oslo in 1995, discussed costs of testing. A final figure was not forthcoming, but it was clear from the delegates present that the methylation test using DNA probes grown in-house was cheaper than a FISH test using commercial probes (authors' unpublished data). In our laboratory, the costs are similar for both tests.

The cost-effectiveness is another matter. Monaghan et al. (1997) show how this is related to the likelihood of the patient actually having the condition for which he or she is referred. They suggested a questionnaire, to enhance the pick-up rate. The clinical acumen of the referring doctors is important, and continuing education is always a priority, but in cases of genuine doubt it seems worthwhile to order the test. In our setting, clinical input was available and useful in evaluating the patient but was more time consuming than initially imagined. It would be simpler if two specimens of blood were collected from the patient—one for cytogenetics and FISH and one for DNA studies. The results could be correlated later. This would involve an additional collection cost and would defeat the aim of logical test progression on a single blood sample, but it would reduce the logistical costs considerably.

In conclusion, we consider the actual costs of the laboratory reagents to be immaterial. The staffing and organizational costs are the major determinant in cost-effectiveness. The recommendations of The American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee (1996)—namely, to make the maximum use of your resources and to remain flexible—are eminently sensible. This is now our policy.

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Further Comments on the Characterization of Founder Amerindian Mitochondrial Haplotypes

To the Editor:

We have detected the presence of 8–10 founder mitochondrial haplotypes in extant Amerindian populations (Bailliet et al. 1994; Bianchi et al. 1995), and we identified four of these haplotypes by use of the acronyms "A_1," "A_2," "D_1," and "D_2." Easton et al. (1996) confirmed most of our findings and used the nomenclature system proposed by us.

In the October 1996 issue of the *Journal,* Forster et al. (1996) verify that the number of ancestral Amerindian mitochondrial haplotypes is greater than the four variants reported by Torroni et al. (1992); and they too use the symbols A_1, A_2, D_1, and D_2 for identification. However, in their report, these authors state that "the decision taken by Bailliet et al. (1994) and Bianchi et al. (1995) to identify founding haplotypes according to 16517 *Hae*III status is therefore phylogenetically questionable, and we do not recommend the use of their haplogroups or their nomenclature" (Forster et al. 1996, p. 938). Moreover, in the legend to their figure 1, Forster et al. (1996, p. 938) say that "the nomenclature used here is not related to that of Bailliet et al. (1994)." In light of these two comments, we think that it is worthwhile to assess the soundness of Forster et al.'s arguments.

The A_1/A_2 and D_1/D_2 haplotypes used by Forster et al. (1996) are differentiated by a base substitution in mtDNA positions 16111 and 16271, respectively. On the other hand, our subgroups A_1/A_2 and D_1/D_2 depend on a base substitution at position 16519. Thus, in both reports haplotype grouping depends on a single transition. Forster et al. (1996) indicate that the T→C transition used by us is recurrent and, according to them, unsuited for phylogenetic use. Yet, the same authors, despite finding that transitions at positions 16325 and 16362 are also recurrent, use them for haplotyping and phylogenetic reconstruction (Forster et al. 1996). Moreover, Torroni et al. (1993), in table V of their report, show that 3 of the 11 cases of Amerindians/Asians having the founder haplotype B exhibit also the T→G transition at position 16111, which is a clear demonstration of recurrence for this site. This is not surprising. Recently, Howell et al. (1996) made a direct appraisal of the mutation rate in human mtDNA and found an average of one nucleotide substitution every 25 generations. Accordingly, if this finding is confirmed, most mitochondrial mutations would be expected to be recurrent.

Transitions at positions 16111 and 16271 produce the loss of a *Cac*81 and a *Bfa*I site, respectively. By analyzing the data in the literature and by restricting a series of A_1/A_2 and D_1/D_2 haplotypes by use of the aforementioned enzymes, we have determined that 92%–95% of these haploforms can be identified as indicated in table 1. The data in table 1 maintain the nomenclature system proposed by us (Bailliet et al. 1994) and used by Forster et al. (1996) but give a better definition of the haplotypes.

Table 1

Characterization of A_1/A_2 and D_1/D_2 Mitochondrial Haplotypes

	Status for Restriction Enzyme (Position)[a]		
Haplotype	*Hae*III (663)	*Cac*81 (16111)	*Hae*III (16517)
A_1	+	+	+
A_2	+	−	−
	*Alu*I (5176)	*Bfa*I (16271)	*Hae*III (16517)
D_1	−	+	+
D_2	−	−	−

[a] A plus sign (+) denotes presence, and a minus sign (−) denotes absence.