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ORIGINAL COMMUNICATION

Multi-gene testing in neurological disorders showed an improved diagnostic yield: data from over 1000 Indian patients

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Abstract

Background Neurological disorders are clinically heterogeneous group of disorders and are major causes of disability and death. Several of these disorders are caused due to genetic aberration. A precise and confirmatory diagnosis in the patients in a timely manner is essential for appropriate therapeutic and management strategies. Due to the complexity of the clinical presentations across various neurological disorders, arriving at an accurate diagnosis remains a challenge.

Methods We sequenced 1012 unrelated patients from India with suspected neurological disorders, using TruSight One panel. Genetic variations were identified using the Strand NGS software and interpreted using the StrandOmics platform.

Results We were able to detect mutations in 197 genes in 405 (40%) cases and 178 mutations were novel. The highest diagnostic rate was observed among patients with muscular dystrophy (64%) followed by leukodystrophy and ataxia (43%, each). In our cohort, 26% of the patients who received definitive diagnosis were primarily referred with complex neurological phenotypes with no suggestive diagnosis. In terms of mutations types, 62.8% were truncating and in addition, 13.4% were structural variants, which are also likely to cause loss of function.

Conclusion In our study, we observed an improved performance of multi-gene panel testing, with an overall diagnostic yield of 40%. Furthermore, we show that NGS (next-generation sequencing)-based testing is comprehensive and can detect all types of variants including structural variants. It can be considered as a single-platform genetic test for neurological disorders that can provide a swift and definitive diagnosis in a cost-effective manner.

Keywords Neurological disorders · Genetic testing · Next-generation sequencing · Multi-gene panel

Introduction

Neurological disorders are one of the major causes of disability and death worldwide. In India alone, about 30 million people are estimated to be affected with neurological disorders [1]. A significant proportion of these are 'neurogenetic disorders', which include disorders, such as epilepsy, ataxia, neuropathy, sensory impairment, myopathy, movement disorders, intellectual disability and others, that are mostly caused due to defects in one or more genes [2]. The burden of neurological disorders on the Indian healthcare

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system has been steadily increasing, thus stressing the need for cost-effective methods for screening and accurate clinical diagnosis of these disorders.

An accurate diagnosis of a neurological disorder becomes essential to design appropriate treatment and management strategies [3]. However, establishing a diagnosis for many of these neurological conditions is a complex, lengthy and expensive process, which starts with recognition of specific phenotypic features and may involve multiple tests followed by consultation with multiple medical specialists. Further, the complexity in diagnosis is compounded by the fact that mutations in the same gene can cause varied clinical presentations; in contrast, many common neurological phenotypes could result from mutations in different genes. Additionally, several conditions show variable expressivity and reduced penetrance, further complicating genetic diagnosis. For these reasons, effective strategies are required to achieve accurate genetic diagnosis for the neurological disorders [4].

Recent advances in genomic technologies such as next-generation sequencing (NGS) have revolutionized the ability to simultaneously sequence and analyze multiple genes associated with various disorders at a significantly lower cost as compared to the traditional methods, thus allowing us to arrive at a genetic diagnosis in a fast, efficient and cost-effective manner. Additionally, NGS is inherently unbiased and limitless with respect to what genes should be evaluated and is less dependent on a priori clinical information [5, 6]. NGS can also potentially detect all types of variants in a single test, such as SNV (single-nucleotide variant), indel (small deletion, duplication and insertion) and structural variants, such as large deletion and duplication; thus, it can serve as a single-platform test for genetic diagnosis. For these reasons, it is becoming widely used in the clinical practice [7, 8].

In India, very few studies have been conducted to determine the prevalence of genetic mutations in neurological disorders. These studies are either done on a relatively small cohort size [9–11] or done for a limited set of genes associated with specific neurological disorders [12–14]. However, a large-scale study evaluating the contribution of various genes across the spectrum of neurological conditions has not been carried out for the Indian population. To accurately determine the genetic load in India for neurological disorders and to address the genetic heterogeneity involved, more comprehensive studies examining a wider mutational spectrum in multiple genes in large cohorts are required. In the current study, we used an NGS-based approach to screen genes associated with various neurological disorders in an Indian cohort (1012 unrelated individuals/families) highlighting the importance of multi-gene-based testing as a comprehensive, single-platform test for arriving at a decisive diagnosis for clinically heterogeneous neurological conditions.

Methods

Patients

This study comprises of 1012 unrelated patients with an indication of neurological disorder, referred to our laboratory which is accredited to CAP (Accredition # 8750941) and NABL (ISO15189) (Certificate No. MC-2434) since 2015. The patients were referred from various hospitals/clinics from across India, between January 2015 and April 2018. Informed consent was obtained from all subjects. In cases where patients were pediatric, informed consent was obtained from the parent or legal guardian of the patient. Sequencing of patient samples was approved by Institutional Ethics Committee of Strand Life Sciences. Majority of the referred patients were pediatric patients, of which 21% (215/1019) were < 1 year and 54% were 2–9 years of age (Figure S1).

Library preparation, multi-gene panel sequencing, data analysis and variant interpretation

DNA was extracted from blood/saliva samples and 50 ng of input DNA was used for NGS using TruSight One Sequencing Panel (Illumina, USA) that contains coding exons and flanking intronic sequences of > 4600 genes associated with inherited Mendelian diseases. Genomic DNA was used for the library preparation for NGS as previously described [15] and sequenced on the NextSeq platform (Illumina) according to the manufacturer's instructions. Detailed information on library preparation and sequencing is provided in the online supplementary information. Variations were identified using the STRAND® NGS software and interpreted on the StrandOmics™ platform, as previously described [15]. The variants identified were classified according to the ACMG (American College of Medical Genetics and Genomics) recommendation for standards for interpretation and reporting of sequence variations [16].

Copy number variation (CNV) analysis

In addition to single-nucleotide variants (SNVs) and small indels, copy number analysis was performed to identify large deletions or duplications ranging from a single exon, multi-exons, whole gene, to multiple genes (continuous) as described previously [15]. Details of the criteria used to assess the quality of the variant along with NGS quality parameters are provided in online supplementary resource 1 and online supplementary resource 2 (Tables S1, S2). To determine if the identified CNV would require a second method validation, the CNVs were assessed based on

their copy number (CN) values and *Z* scores. Following cutoffs were considered to call out copy number variations: single exon cases, deletions: CN < 1.2, *Z* score > 7; multi-exon cases, deletions: CN < 1.2, *Z* score > 5; duplications: CN > 2.8, *Z* score > 5, multi-gene cases: deletions: CN < 1.3, *Z* score > 4; duplications: CN > 2.7, *Z* score > 4 in the gene of interest. The CNV calls which did not meet the above-mentioned CN and *Z* score cut-off values were validated by quantitative polymerase chain reaction (qPCR) (Table S2). To identify breakpoints where possible, split read alignment was performed on the reads that did not align with an alignment score > 95%, as described previously [17].

Second method validation of mutations detected by NGS data

To evaluate the accuracy of the NGS variant calling pipeline in calling out high-confidence variants and to determine if the identified SNVs and indels would require second method validation, we followed a protocol previously described [18]. Details of the criteria used to assess the quality of the variant along with NGS quality parameters are provided in online supplementary resource 1 and online supplementary resource 2 (Tables S1, S2). To determine if the identified SNVs and indels would require second method validation, all the variants were assessed for several quality parameters. The parameters considered include supporting reads (SR) for the variant call, total reads (TR), strand bias (SB) for the variant, base quality (BQ) and mapping quality (MQ). Previously, a concordance study performed on over 20,000 samples for 7845 variants showed that all variants with coverage > 100× and a SR > 40% could be confirmed by Sanger [19]. In another study by Baudhuin et al., 2015, it was shown that variants with coverage > 100× and a quality score Q > 20 could be confirmed by Sanger and thus, performing Sanger for variants called out by NGS testing that meets appropriate quality thresholds is unnecessarily redundant [20]. Similar finding has also been reported by Nelson et al. (2015), where they observed 100% concordance between the NGS variant call and Sanger validation, upon using appropriate quality criteria (Nelson et al. 2014). Moreover, multiple large-scale studies suggest that majority of clinically reported variants could be confidently released without Sanger confirmation [21, 22]. Thus, following cut-off values were considered for reporting variants without confirmation by Sanger sequencing: SR% ≥ 30 for heterozygous variant and ≥ 90 for homozygous variant, TR ≥ 40 for heterozygous and ≥ 20 for homozygous variant, SB ≤ 30, BQ ≥ 30 and MQ ≥ 250. Sanger validation was also considered for variants mapping to the low complexity region such as a homopolymer (< 7 bases) or repeat region. However, as with any other molecular tests, there is a small chance that this result may be inaccurate due to the technical limitations. Since the mutations identified by NGS have not been confirmed by a second test such as Sanger sequencing, there is still a possibility of the finding being false positive.

Results

Mutation detection rate in the cohort of patients with neurological disorders

In the 1012 samples tested in this study, we detected a total of 455 pathogenic/likely pathogenic variants in 197 genes. Of these, 178 were novel variants (Tables S3, S4). In 405 patients (40%), a definitive diagnosis could be arrived at, wherein causative variant(s) in a clinically relevant gene associated with the referred clinical indication was identified (Fig. 1a, Tables S3, S4). In 4.4% (45/1012) of the cases, the reported variant(s) was categorized as 'variants of uncertain significance with probable damaging effect' (VUSD) (Table S5). These variants are likely to contribute to the disease; however, further scientific or clinical evidence would be required to establish this conclusively. In addition, in 25% (257/1012) of total cases, we detected only 'variants of uncertain significance' (VUS) in clinically relevant genes (Table S6).

In 21 cases (2%), the diagnosis was 'inconclusive' wherein, a single heterozygous pathogenic or likely pathogenic variant was detected in a clinically relevant gene for an autosomal recessive (AR) condition and the second disease-causing variant could not be identified (Table S3). Possibility of the second disease-causing variant in deep intronic or promoter region or any complex structural variants cannot be ruled out in these cases.

In addition to the diagnostic findings, 12 of the 1012 patients had a secondary finding (unrelated conditions as per ACMG guidelines of reporting) [16] (Table S7).

Pattern of inheritance in cases with confirmed diagnosis

Among the 405 affected individuals who received a confirmed diagnosis, based on the mode of inheritance associated with the genes and zygosity of the variant, we could categorize 222 (~ 55%) patients as those affected with an AR disorder, 114 (~ 28%) patients with an autosomal dominant condition and 69 (~ 17%) patients with an X-linked disease (Tables S3, S4). In patients with an AR disorder, 167 patients harbored a homozygous variant, while 55 harbored 2 heterozygous variants. In the latter case, 2 pathogenic/likely pathogenic variants were detected in 25 individuals; while in 30 individuals, the second variant was a VUSD or a VUS (Table S3).

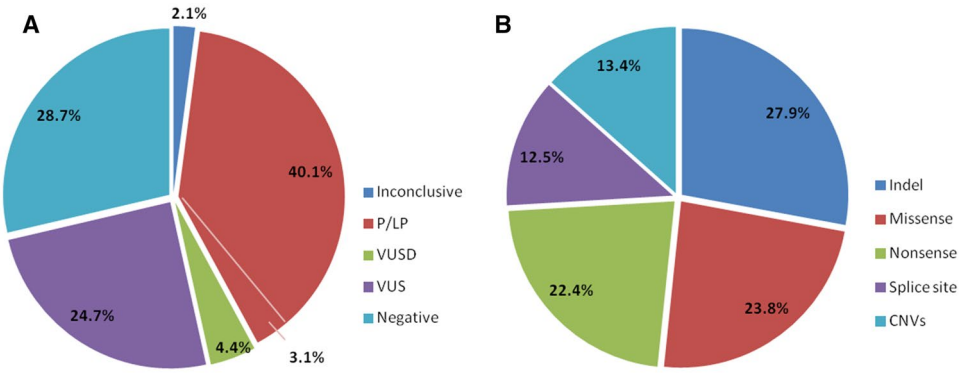

Fig. 1 Mutation detection rate and mutation spectrum in neurological disorders cohort. **a** The pie chart shows mutation detection rate of 40.1% (405) (which includes 'pathogenic' (P) and 'likely pathogenic' (LP) variants in 37% of cases, and in 3.1% cases, a VUSD/VUS was identified in addition of a P/LP variant). 'Variants of uncertain significance with probable damaging effect' (VUSD) were identified in 4.4% (45) and VUS were identified in 24.8% (251) of cases, while no mutation was detected in 28.7% (290) of cases. 2.1% (21) cases were inconclusive, where we could identify only one P or LP variant in an autosomal recessive condition. **b** The mutation types detected in our cohort were indel (small deletion, duplication, insertion, or insertion/deletion), missense, nonsense, splices site, CNVs (large deletion, large duplication and complex rearrangement). Overall, 127 (27.9%) indel, 108 (23.8.0%) missense, 102 (22.4%) nonsense, 57 (12.5%) splice site, and 61 CNVs (13.4%) (54 large deletions, 6 large duplications and 1 complex rearrangement) were detected

Mutation spectrum in the cohort

The spectrum of identified variants included 127 (27.9%) indel (small deletion, duplication, insertion, or insertion/deletion), 109 (24.0%) missense, 101 (22.2%) nonsense, 57 (12.5%) splice site and 61 (13.4%) structural variants (large deletion/duplication) (Fig. 1b). None of these variants (in relevant zygosity) were found in > 2500 control individuals sequenced in our laboratory (The data of control samples used in this study are available in the following url link: https://khasi.strandls.com/pub-data/ControlVCFfile/).

Identification of structural variations

We identified 61 structural variants in our cohort, which accounted for 15% of the patients who received definitive diagnosis. The spectrum of structural variants identified in our cohort ranged from single exon, multi-exons, whole gene, to multiple genes (contiguous). Deletions were detected in 54 patients; duplications were detected in six patients; and in one patient, complex rearrangement was observed (Table S4). In the patients in whom structural variants were detected, 33 patients were those who were referred with multiple neurological phenotypes. Among the clinical subtypes, the highest frequency of structural variants was detected in epilepsy (14%; 13 out of 90) and muscular dystrophy (14%; 10 out of 70) followed by microcephaly (6%, 6 out of 92) (Table S4).

Mutation detection rate in the clinical subtypes

The mutation detection rates observed in various clinical subtypes of neurological disorders in our cohort ranged from 25 to 65%. The highest diagnosed yield was observed in muscular dystrophy (64%), followed by leukodystrophy (43%), ataxia (43%), myopathy (42%) and neuropathy (39%) (Table 1). Among patients with epilepsy, which was the most commonly occurring clinical indication in our cohort (292 patients), the diagnostic yield was 31%.

Several genes showed recurrent mutations in our cohort. The *DMD* gene (22/405; 13.1%) was the most recurrently mutated gene and other recurrently mutated genes in this cohort were *MECP2*, *SCN1A*, *LAMA2*, *CAPN3* and *CHRNE* (Fig. 2).

Diagnosis in cases with complex neurological phenotypes

Among the patients who were referred for testing, a significant proportion of those were referred with no suggestive diagnosis. Based on symptomatic assessment, they were suspected to have a neurological disorder. 106 such patients received a confirmatory diagnosis after the testing, accounting for 26% of the patients who received definitive diagnosis (106/405). 33 patients among them harbored a structural variant. Notably, 61 structural variants were identified in our cohort, and 55% were those who manifested multiple neurological phenotypes. Additionally, there were 22 patients in whom a suggestive diagnosis was provided but, upon testing,

Table 1 Mutation detection rate in clinical subtypes of neurological disorders in the study

Clinical subtypes	No. of cases	P + LP[a]	VUSD[a]	VUS[a]	Negative[a]	Inconclusive[b]
Epilepsy	292	30.8	5.1	33.2	28.1	2.7
Muscular dystrophy	107	65.4	2.8	20.6	10.3	0.9
Autism spectrum disorders	101	35.6	1.0	23.8	38.6	1.0
Microcephaly	92	38.0	0.0	21.7	33.7	6.5
Ataxia	88	43.2	5.7	25.0	26.1	0.0
Neuropathy	68	39.7	13.2	23.5	20.6	2.9
Leukodystrophy	66	43.9	3.0	31.8	19.7	1.5
Intellectual disability	62	29.0	3.2	33.9	32.3	1.6
Myopathy	45	42.2	2.2	35.6	15.6	4.4
Developmental delay	44	31.8	2.3	15.9	47.7	2.3
Spasticity	43	34.9	4.7	25.6	32.6	2.3
Dystonia	41	26.8	4.9	22.0	41.5	4.9
Others	151	45.0	5.3	18.5	29.8	1.3

P pathogenic, *LP* likely pathogenic, *VUSD* variant of uncertain significance with probable damaging effect, *VUS* variant of uncertain significance

[a]Values represented in percentage

[b]Percentage of cases in which only a P or LP variant was detected in an autosomal recessive condition

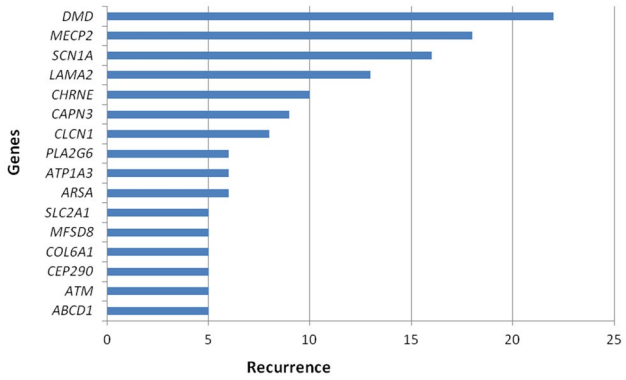

Fig. 2 Gene recurrence. The most frequently mutated gene was *DMD* (22). Other recurrently mutated genes include *MECP2* (18), *SCN1A* (16), *LAMA2* (13), *CHRNE* (10), *CAPN3* (9), *CLCN1* (8), *ARSA*, *ATP1A3*, and *PLA2G6* (6 each), *ABCD1*, *ATM*, *CEP290, COL6A1, MFSD8*, and *SLC2A1* (5 each). Other genes showed recurrence < 5 (not included in the figure)

a pathogenic or likely pathogenic variant was detected in a gene associated with a different condition that clinically overlaps with the suspected condition (Table S8).

Discussion

We performed multi-gene testing on a cohort of 1012 patients with neurological disorders and observed a diagnostic yield of 40%. In addition, in 4.4% of the cases, we observed VUSD, which are potentially disease-causing variants. The diagnostic yield for neurological disorders observed in this study is higher than several other large-scale studies in which a significant proportion of the patients screened were affected with neurological disorders and the detection rate was in the range of 25–35%, among the neurological patients [21, 23–25].

In our cohort, we have further categorized the neurological disorders into different disease groups and highest numbers of patients were referred for epilepsy. The diagnostic yield for epilepsy was 31% in our study. In a recent study done on > 8000 epilepsy patients, detection rate of 15.4% was reported [26]. Other large-scale studies also indicate a detection rate of 10–20% in patients with epilepsy [27, 28]. Similarly, in our cohort, a higher diagnostic yield has also been observed for other conditions, such as ataxia, muscular dystrophy and myopathy as compared to other studies [29–31]. One of the reasons for the observed higher detection rate in our study could be due to referral bias, where it is possible that those patients with neurological condition with a strong likelihood of genetic cause were referred to our laboratory. Another reason could be due to a high number of consanguineous and endogamous marriages practiced in India, which significantly increase the risk for recessive disorders.

Chromosomal microarray (CMA) has been recommended as the first-tier test in patients manifesting developmental delay, autism and intellectual disability, with a diagnostic yield of 15–20% [32]. In our cohort, in patients who manifested global developmental delay, we observed a detection rate of 34% (15/46) and 46.6% mutations were structural variants. It is important to mention that the sample size (46 patients) in our study is relatively small and further studies with higher numbers will be needed to substantiate this finding. Interestingly, in our cohort, among the pathogenic/likely

pathogenic variants detected, 13.4% were structural variants and 49.2% (30/61) of these variants were cytogenetic band deletions/duplications, which are typically detected by CMA or fluorescent in situ hybridization (FISH). Recent studies have also shown that NGS-based tests can reliably detect structural variants by employing high-resolution exon level copy number detection methods in the pipeline [18, 33, 34].

Neurological disorders are clinically heterozygenous group of disorders. In order to reach to a diagnosis, multitude of tests is performed on the patients. In a study done on a cohort of 150 patients and parents, who received both the standard diagnostic workup (e.g., cerebral imaging, muscle biopsies or lumbar punctures, and sequential gene-by-gene testing) and whole-exome study (WES) simultaneously, it was observed that the WES identified significantly more conclusive diagnoses (29.3%) than the routine diagnostic tests (7.3%) without incurring higher costs [4]. Our study also reinforces the importance of NGS-based testing in differential diagnosis of neurological conditions. We were able to arrive at a 'conclusive' diagnosis in 26% of the cases, which are associated with known syndromes; however, conventional clinical investigation had failed to arrive at the precise diagnosis in these cases. In a significant proportion of patients, our NGS-based test helped in arriving at a precise clinical subtype of the disease, such as Dravet syndrome, Duchene muscular dystrophy and neuronal ceroid lipofuscinosis, which facilitated personalized clinical management.

Our targeted NGS panel comprised of > 4500 genes associated with Mendelian disorders. However, we detected a pathogenic/likely pathogenic variant only in 197 genes, in our study. Of the 197 genes, 18 genes alone accounted for diagnosis in ~ 40% of the cases. In a recent study, Lindy and colleagues analyzed 70 well-known genes associated with epilepsy and screened > 8000 patients. However, only 22 genes showed high diagnostic yield [26]. In large-scale WES studies done on 500–1000 patients with neurological disorders, pathogenic variant have been reported in ~ 600 genes. Individually, each study reported pathogenic variants in 150–400 genes [22, 23, 25]. Multiple large-scale studies on complex neurological indications have reported a diagnostic yield between 25 and 35% for WES, and between 30 and 35% for whole-genome studies (WGS) [25, 35–37]. In our study, we reported a diagnostic yield of 40% in 197 genes in a cohort of > 1000 patients. It is, thus, evident that WES, WGS and multi-gene panel with large number of genes do not necessarily implicate an improved diagnostic yield compared to a targeted panel covering only genes associated with the relevant disorder(s).

In a clinical setting, for a set of diseases/clinical phenotypes under consideration, a NGS-based multi-gene panel, which is robustly validated for all test parameters, may offer the maximum diagnostic yield. Also, as the cost of the test is directly proportional to the number of genes present in a test, the substantially low numbers of the genes present in a targeted panel can allow us to achieve a very significant cost reduction. In developing countries, such as India, availability of such cost-effective test is crucial for ensuring that it is accessible to majority of the patients.

Our study shows an improved performance of multi-gene panel testing, with an overall diagnostic yield of 40%. It also highlights that the NGS-based test is comprehensive that can detect all types of mutations and will be cost effective when compared to reflex mode testing using conventional methods or WES. In conclusion, data from our study strongly suggest that the NGS-based multi-gene test should be considered as the first-tier genetic test for neurological disorders in India.

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Author contributions AG—acquisition of data, analysis of data, interpretation and critical review of data, drafting of manuscript. AV, MRS—acquisition of data, analysis of data, interpretation of data, making of figures. PK, MS, AVK, IRPP, SG, AS, TTC, ASL, VHK, SMC—acquisition of data, interpretation of data. VR, SN, RM, DP, VU, NN, MK, ARRD, MK, SJ, MN, AUH, MPP, SS, PT, RP, AH, KS, JS—acquisition of data and concept of the study. PA—acquisition of data, sequencing. SK, VV—analysis and validation. AM, AG—conception and design of study, analysis of data, interpretation and critical review of data, drafting of manuscript. AM, VC, RH—critical inputs and finalization of the manuscript. All authors contributed in preparation of the manuscript, read and approved the final manuscript.

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Compliance with ethical standards

Conflicts of interest AG, AV, MRS, PK, MS, AVK, IRPP, SG, AS, TTC, ASL, VHK, SMC, SN, MBP, VGR, MP, PA, SK, VV, VC, RH, AM are employees of Strand Life Sciences that offer commercially available clinical genetic testing services.

Ethical standards Sequencing of patient samples used in the study has been approved by Institutional Ethics Committee of Strand Life Sciences.

Informed consent Informed consent was obtained in writing from all subjects and sequencing of patient samples was approved by Institutional Ethics Committee of Strand Life Sciences.

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