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Evaluation of the GenoType MTBDR*plus* assay for detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates in central Ethiopia ☆

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ABSTRACT

Objective/Background: Multidrug-resistant tuberculosis (MDR-TB) is growing globally and becoming a major challenge for national TB control programs. Therefore, rapid identification of MDR strains of *Mycobacterium tuberculosis* and monitoring their transmission could contribute significantly to the control of TB. The GenoType MTBDR*plus* assay has been recommended by the World Health Organization to identify rifampicin (RIF)- and isoniazid (INH)-resistant *M. tuberculosis* isolates. This study was carried out to evaluate the performance of the GenoType MTBDR*plus* assay for the detection of RIF- and INH-resistant *M. tuberculosis* isolates in central Ethiopia. *Methods:* A total of 279 *M. tuberculosis* strains isolated from active TB cases in central Ethiopia were evaluated for their drug sensitivity by the conventional drug-susceptibility test (DST) and compared with data derived from the GenoType MTBDR*plus* assay. The DST served as the gold standard for evaluating the GenoType MTBDR*plus* assay. *Results:* The sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of RIF-resistant *M. tuberculosis* isolates were 80.0% and 99.6%, respectively. Its sensitivity and specificity for the detection of INH-resistant *M. tuberculosis* isolates were 82.7% and 99.6%, respectively, whereas they were 75.0% and 100%, respectively, for the detection of MDR *M. tuberculosis* strains. The concordances of the GenoType MTBDR*plus* assay and the conventional DST for the detection of RIF and INH susceptibility were 80% (8/10) and 86.2% (25/29), respectively. Furthermore, the concordance of the two tests for the detection of MDR *M. tuberculosis* strains was 75%. Specific mutations were detected in 55.6% (5/9) of the RIF-resistant isolates, with the highest mutation rate (33.3%) for the *rpoB* gene (Codon S531L). For INH-resistant isolates, the highest mutation

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rate (88.8%) related to a *katG* mutation (Codon S315T1). *Conclusion:* The findings of this study revealed that the GenoType MTBDR*plus* assay has high sensitivity and specificity for the detection of RIF and INH resistance. These preliminary data support the notion that the assay should be considered as an alternative to the DST for the characterization of MDR in *M. tuberculosis* isolates and the control of TB.

Introduction

Tuberculosis (TB) is one of the major global health problems, with high prevalence in developing countries. A major concern is multidrug-resistant TB (MDR-TB), which is defined as the resistance to isoniazid (INH) and rifampicin (RIF), two therapeutic compounds for first-line TB treatment. The emergence of strains resistant to major anti-TB drugs has increased the need for identifying rapid and simple methods to detect such resistances and their molecular basis in the *Mycobacterium tuberculosis* genome. Using such tests promises to improve the physician's decision to appropriately treat the disease in patients on an individual basis and allows better monitoring of the emergence of MDR-TB strains in distinct geographical regions, ultimately contributing to the prevention of the spread of resistant strains. Drug-susceptibility testing by conventional methods using solid media such as Löwenstein–Jensen is time consuming because *M. tuberculosis* grows slowly in culture requiring several weeks to identify the pathogen and test its drug-resistance profile. Even with more automated fluid culture methods, the former method takes an average of 14 days. Two additional weeks are required to obtain information about the strain's drug susceptibility [1]. Molecular methods for drug-resistance testing based on the identification of mutations in genes associated with drug resistance, such as the GenoType MTBDR*plus* assay, offer an effective, alternative method to determine drug-resistance strains [2]. The GenoType MTBDR*plus* assay is a molecular-line probe assay containing probes specific for the *M. tuberculosis* complex, wild type as well as probes for common RIF- and INH-resistance-conferring mutations. The assays are based on reverse hybridization of amplicons immobilized on membranes. The GenoType MTBDR*plus* assay detects mutations in the *rpoB*, *katG*, and *inhA* genes, and delivers results with a rapid turnaround time of 48–72 h. Nearly all RIF-resistant strains contain mutations in the *rpoB* gene, which encodes the RNA polymerase subunit β [3]. Mutations in the *katG* and *inhA* genes are related to the high-level and low-level INH resistance, respectively [3]. More than 95% of the RIF-resistant strains harbor a mutation within an 81-bp region of the *rpoB* gene from Codons 507 to 533 and this is region is called the RIF resistance-determining region [4–6]. The highest level of RIF resistance of the *rpoB* gene occurs in Codons 531 and 526. The *rpoB* gene mutations occur in Codons 511, 516, 518, 522, and 533 and cause low-level resistance to RIF. Resistance mutations are rarely identified in other regions of the *rpoB* gene [4].

Mutations causing INH resistance are located in several genes. Several studies have demonstrated that 34.6–94.3% of INH resistance is most frequently associated with a mutation in Codon 315 of the *M. tuberculosis* catalase peroxidase (*katG*) gene [7,8]. The *inhA* gene has 2.9–21.5% of its mutations in the promoter region [9], and an additional 2–11.5% in the *ahpC–oxyR* intergenic region [10,11].

Ethiopia is one of the 27 high MDR-TB-burden countries in the world. According to the 2015 World Health Organization report, 1.6% of new TB patients and 12.0% of previously treated patients had MDR-TB [12]. Annually, 2000–2500 MDR-TB cases are estimated to occur among all reported pulmonary TB cases. However, for example, in the year 2012, only 212 (10.1%) TB cases were detected [13], indicating that the many of the expected MDR-TB cases remain undiagnosed and continue to spread in various communities. Therefore, improved monitoring of TB drug resistance—with respect to the time required for detection of resistance and the sensitivity and specificity of detection of MDR—is important, and may benefit from molecular tests such as the GenoType MTBDR*plus* assay. The objective of this study was to evaluate the performance of the GenoType MTBDR*plus* test in detecting INH- and RIF-resistant *M. tuberculosis* isolates in central Ethiopia.

Materials and methods

M. Tuberculosis isolates

A total of 279 *M. tuberculosis* specimens isolated from smear-positive TB patients who visited St. Lukas, Atat, and Fitche hospitals in central Ethiopia between October 2012 and September 2013 were used for this study. The study was ethically approved by the Ethical Review Board of College of the Natural Sciences of the Addis Ababa University, Ethiopia (Ref. No. CNSDO/379/07/15).

Conventional drug-susceptibility testing using Löwenstein–Jensen media

The isolates were evaluated for their drug sensitivity using the conventional proportion method and the sensitivity of each isolate against the first-line drugs (INH, streptomycin, RIF, and ethambutol) was evaluated by the indirect proportion

method on Löwenstein–Jensen medium, according to an international standard [14].

GenoType MTBDRplus assay

The GenoType MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) was performed according to the manufacturer's instructions [10]. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex polymerase chain reaction (PCR) amplification, and reverse hybridization. The assay screens for the absence and/or presence of wild-type (WT) and/or mutant (MUT) DNA sequences within specific regions of three genes: the *rpoB* gene for RIF resistance, the *katG* gene for high-level INH resistance, and the *inhA* gene for low-level INH resistance. Each strip contains 27 reaction zones and the results can be obtained within a day [11]. In brief, for one PCR, 10 μL amplification mix A containing 10× buffer, nucleotides, and DNA polymerase was mixed with 35 μL of amplification mix B containing $MgCl_2$, the biotinylated primers, and dye. Then, 5 μL of *M. tuberculosis* DNA was added to the mixture, making the final volume of PCR mix to be 50 μL. The PCRs consisted of 15 min of denaturing at 95 °C, followed by 10 cycles of 30 s at 95 °C and 120 s at 58 °C, followed by 20 additional cycles of 25 s at 95 °C, 40 s at 53 °C, and 40 s at 70 °C, with a final extension at 70 °C for 8 min. For hybridization, 20 μL of the amplification products were mixed with 20 μL of the denaturing reagent (provided with the kit) and denaturing was performed for 5 min in each of the plastic well. Thereafter, 1 mL of prewarmed hybridization buffer was added into each well and one strip was placed in each well. The hybridization was performed at 45 °C for 30 min, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin conjugated with alkaline phosphatase was added after which a substrate buffer was added. After final washing, strips were air dried and fixed on paper. The DNA of the standard strain $H_{37}Rv$ and molecular-grade water were used as positive and negative controls, respectively.

Interpretation of results

Each strip consists of 27 reaction zones (bands) with six controls including conjugate control (CC), amplification control (AC), *M. tuberculosis* complex, *rpoB* locus control, *katG* locus control, and *inhA* locus control. The remaining 21 reaction zones are WT and mutation reaction zones including eight *rpoB* WT and four MUT probes, one *katG* WT and two MUT probes, and two *inhA* WT and four MUT probes. Results were interpreted according to the manufacturer's instructions [10]. In brief, the presence of CC bands indicates the efficiency of the conjugate and substrate, the presence of AC bands indicates the efficiency of DNA extraction and PCR procedures, and the presence of the *M. tuberculosis* complex band indicates that the tested bacterium belongs to the *M. tuberculosis* complex. The three respective locus control bands (*rpoB*, *katG*, and *inhA*) indicate the presence of the specific gene region. The absence of the WT band is usually accompanied by the presence of MUT, which indicates resistance and the presence of all WT bands without the MUT band indicates susceptible isolate. In rare cases, lack of WT band(s) without a corresponding MUT band could be observed due to uncommon mutations in the probe region and the presence of both WT and MUT bands in the same stripe might be an indication for the presence of heteroresistance or mixed infection.

Results

Sensitivity and specificity of the GenoType MTBDRplus assay for detection of RIF- and INH-resistant M. tuberculosis

Drug-sensitivity test was conducted on 279 *M. tuberculosis* isolates using the GenoType MTBDR*plus* assay and the Löwenstein–Jensen medium-based proportion method. The result of the GenoType MTBDR*plus* assay showed that most isolates (96.8%) were susceptible to RIF; by contrast, susceptibility to INH (91.0%) was lower than that of RIF (Table 1). The GenoType MTBDR*plus* assay detected nine (3.2%) RIF-resistant and 25 (9.0%) INH-resistant isolates. Moreover, three (1.1%) isolates were found to be MDR by the GenoType MTBDR*plus* assay.

The results of the evaluation of the performance of the GenoType MTBDR*plus* assay in detecting drug resistance are summarized in Table 2. The sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of RIF-resistant *M. tuberculosis* isolates were 80.0% and 99.6%, respectively. Its sensitivity and specificity for detecting INH-resistant *M. tuberculosis* isolates were 82.7% and 99.6%, respectively, whereas they were 75.0% and 100%, respectively, for detecting MDR *M. tuberculosis* strains. The concordances of the GenoType MTBDR*plus* assay and the conventional DST for the detection of RIF and INH susceptibility were 80% (8/10) and 86.2% (25/29), respectively. Furthermore, the concordance of the two tests in detecting MDR *M. tuberculosis* strains was 75%.

Mutation patterns of RIF and INH produced by the GenoType MTBDRplus assay

Specific mutation was detected in five of the nine RIF-resistant isolates. Of these five, three isolates had mutation at Codon S531L, whereas the remaining two isolates had mutation at Codon H526D. In the remaining four RIF-resistant isolates, WT8 probe was missing with no gain in MUT3 probes and considered "unknown."

In the GenoType MTBDR*plus* assay, INH resistance was detected by using the probes of the *katG* and *inhA* genes. Of the 25 INH-resistant isolates, *katG* mutation occurred in 88.0% (22/25) of the isolates and in all of these isolates, specific mutations were observed at Codon S315T1 of the *katG* gene. Mutations in the *inhA* gene occurred only in three INH-resistant isolates. Specific *inhA* mutations were observed in two of the INH-resistant isolates, which had mutation at Codon C15T, whereas in the remaining one isolate, the *inhA* WT2 gene was missing without the presence of specific mutation band. These isolates are also considered unknown (Table 3).

Band patterns of drug-resistant M. tuberculosis strains

The genotypic profile of resistance to RIF and NIH was examined. Of the nine isolates that showed resistance to RIF by the

Table 1 – Drug-susceptibility test result of the GenoType MTBDR*plus* assay (*n* = 279).

Drug-resistance pattern	New cases *n* (%)	Treated cases *n* (%)
RIF susceptible	260 (97.0)	10 (90.9)
INH susceptible	243 (90.6)	11 (100)
Only RIF resistance	5 (1.9)	1 (10.0)
Only INH resistance	22 (8.2)	0 (0.0)
Any RIF resistance	8 (3.0)	1 (10.0)
Any INH resistance	25 (9.3)	0 (0.0)
MDR (RIF + INH) resistance	3 (1.1)	0 (0.0)

INH, isoniazid; MDR-TB, multidrug-resistance tuberculosis; RIF, rifampicin.

Table 2 – Performance of the GenoType MTBDR*plus* assay and the LJ medium-based proportion method as a gold standard for the detection of resistance of *Mycobacterium tuberculosis* to rifampicin and isoniazid.

GenoType MTBDR*plus* assay		LJ DST result					
		Resistance	Susceptible	Sensitivity	Specificity	PPV	NPV
RIF	Resistant	8	1	80	99.6	88.9	99.2
	Susceptible	2	268				
INH	Resistant	24	1	82.7	99.6	96	98.4
	Susceptible	5	249				
MDR	Resistant	3	0	75	100	100	99.6
	Susceptible	1	275				

DST, drug-susceptibility testing; INH, isoniazid; LJ, Löwenstein–Jensen; MDR, multidrug resistance; NPV, negative predictive value; PPV, positive predictive value; RIF, rifampicin.

GenoType MTBDR*plus* assay, the missing of WT5 was observed in two isolates, but missing of WT8 was observed in seven isolates. MUT2A was observed in two RIF-resistant isolates and MUT3 was observed in three RIF-resistant isolates. In the remaining four isolates, WT8 was missing without the corresponding mutation of MUT3.

Of the 25 INH-resistance isolates, 88% (22/25) isolates had a high-level resistance profile to the drug, which indicated absence of WT band WT1 and presence of corresponding mutation bands of MUT1 in the *katG* gene. The remaining three isolates showed a low-level resistance pattern; WT1 was missing in two isolates and WT2 was missing in one isolate of the *inhA* gene in INH resistance, and there was a corresponding mutation in MUT1 in two isolates of the *inhA* regulatory region (Fig. 1).

The band patterns of isolates resistant to RIF and INH were observed in six of the RIF mono-resistant, 22 of the INH mono-resistant, and three MDR isolates. In the *rpoB* gene, the absence of WT5 was observed in two RIF mono-resistant isolates and there was a corresponding MUT2A in these two RIF mono-resistant isolates. The absence of WT8 was observed in four RIF mono-resistant isolates and in all of the three MDR isolates. The corresponding MUT3 was observed in only one RIF mono-resistant and in two MDR isolates. In the remaining four RIF-resistant isolates, WT8 probe was missing with no gain in MUT3 probes (Table 4).

In the case of INH-resistant strains, in the *katG* gene, the missing WT1 was observed in 19/22 (86.4%) of the INH mono-resistant isolates and in all of the three MDR isolates (Table 4). The corresponding *katG* MUT1 was observed in all of these 19 INH mono-resistant isolates and in all of the three MDR isolates. In the case of the *inhA* gene, the absence of WT1 was observed in only two isolates and there was a presence of the corresponding MUT1 in these two INH mono-resistant isolates. In the same *inhA* gene, WT2 was missing in one INH mono-resistant isolate without the presence of the corresponding mutation gene.

Table 3 – Mutation patterns of the GenoType MTBDR*plus* assay.

rpoB mutations	Frequency	*katG* mutations	*inhA* mutations	Frequency
S531L	3	S315T1	WT	22
H526Y	2	WT	C15T	2
UK (WT8 missed)	4	WT	UK (WT2 missed)	1
RIF resistant	9		INH resistance	25
RIF susceptible	270		INH susceptible	254

INH, isoniazid; RIF, rifampicin; UK, unknown mutation characterized by no hybridization to one or more wild-type probes nor to any of mutation probes; WT, wild type.

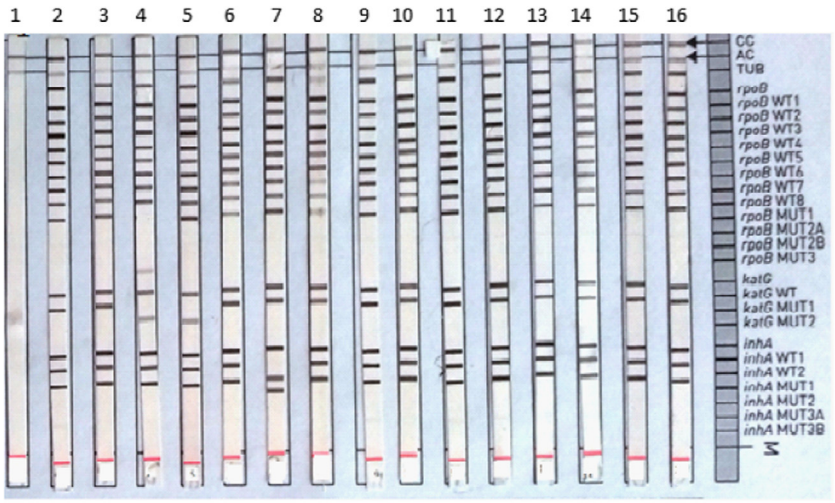

Fig. 1 – Representative DNA patterns obtained by the GenoType MTBDR*plus* assay. Lane 1, water as a negative control, Lanes 2, 3, 6, 8, 9, 10, 11, 12, 13, 14, and 15 all are examples of a pattern of RIF^s and INH^s; Lane 4, example of a pattern of RIF^r and INH^r; Lane 5, example of a pattern of RIF^s and INH^r with *katG* mutation; Lane 7 is an example of a pattern of RIF^s and INH^r with *inhA* mutation; Lane 16, H_{37}Rv as a positive control. INH, isoniazid; RIF, rifampicin. *Note:* The superscript 'r' represent resistance and 's' represent sensitive.

Discussion

In this study, the performance of the GenoType MTBDR*plus* assay for the detection of RIF- and or INH-resistant strains of *M. tuberculosis* was evaluated on 279 *M. tuberculosis* isolates, which were isolated from pulmonary TB patients at three towns and their surroundings in central Ethiopia. We observed that the GenoType MTBDR*plus* assay results had a good concordance with the conventional DST with additional advantage of a shorter turnaround time. The sensitivity and specificity of the GenoType MTBDR*plus* assay for detection of RIF- and INH-resistant *M. tuberculosis* isolates were very good. The overall concordance of the GenoType MTBDR*plus* assay and the conventional DST method was 88.5%. The

Table 4 – Band patterns of drug-resistant *Mycobacterium tuberculosis* strains using the GenoType MTBDR*plus* assay.

Gene	Band	Gene region/mutation	RIF mono resistance (*N* = 6) *n* (%)	INH mono resistance (*N* = 22) *n* (%)	MDR (*N* = 3)
rpoB					
	WT1	506–509	6 (100)	22 (100)	3 (100)
	WT2	510–513	6 (100)	22 (100)	3 (100)
	WT3	513–517	6 (100)	22 (100)	3 (100)
	WT4	516–519	6 (100)	22 (100)	3 (100)
	WT5	518–522	4 (66.7)	22 (100)	3 (100)
	WT6	521–525	6 (100)	22 (100)	3 (100)
	WT7	526–529	6 (100)	22 (100)	3 (100)
	WT8	530–533	2 (33.3)	22 (100)	0 (0)
	MUT1	D516V	0 (0)	0 (0)	0 (0)
	MUT2A	H526Y	2 (33.3)	0 (0)	0 (0)
	MUT2B	H526D	0 (0)	0 (0)	0 (0)
	MUT3	S531L	1 (16.7)	0 (0)	2 (66.7)
katG					
	WT1	315	6(100)	3 (14.5)	0 (0)
	MUT1	S315T1	0 (0)	19 (86.4)	3 (100)
	MUT2	S315T2	0 (0)	0 (0)	0 (0)
inhA					
	WT1	−15/−16	6 (100)	20 (90.9)	3 (100)
	WT2	−8	6 (100)	21 (95.4)	3 (100)
	MUT1	C15T	0 (0)	2 (9)	0 (0)
	MUT2	A16G	0 (0)	0 (0)	0 (0)
	MUT3A	T8C	0 (0)	0 (0)	0 (0)
	MUT3B	T8A	0 (0)	0 (0)	0 (0)

INH, isoniazid; MDR, multidrug resistant; MUT, mutation; RIF, rifampicin; WT, wild type.

sensitivity and specificity of the GenoType MTBDR*plus* assay were 80.0% and 99.6%, respectively, for the detection of RIF resistance. Moreover, they were 82.7% and 99.6%, respectively, for the detection of INH resistance. The result of another study conducted in northwest Ethiopia reported sensitivity and specificity of 92.0% and 99.0%, respectively, for the detection of INH resistance, whereas both sensitivity and specificity for the detection of RIF resistance were 100% [15]. A study in India reported sensitivity, specificity, positive predictive value, and negative predictive value of 97.6%, 94.4%, 97.6%, and 94.4%, respectively, for the detection of RIF resistance, but the same study reported sensitivity and specificity values of 83.3% and 93.8%, respectively, for the detection of INH resistance [16]. Studies in Uganda and France [17,18] reported sensitivity of 100% for the detection of RIF resistance.

In this study, the GenoType MTBDR*plus* assay identified RIF-resistance-specific mutation by *rpoB* MUT probes, which was detected in five of the nine RIF-resistant isolates. Higher specific mutation on the *rpoB* gene was reported in another study in India [19] and in South Africa [20].

The result of this study showed that of the five specific mutations of the *rpoB* gene, three had mutations at Codon S531L. The remaining 22.2% (2/9) had mutation at Codon H526D. A similar finding was reported by a study conducted in India [21]. In four of the nine RIF-resistant isolates, WT8 probe was missing with no gain in MUT3 probes, which indicated the presence of a less common or rare mutation. Similarly, RIF-resistant isolates with the missing of WT8 probe without any MUT band were reported in other studies from New Delhi, France and Vietnam [16,18,22]. Another study conducted in northwest Ethiopia reported that all phenotypically defined RIF-resistant strains and MDR strains had mutations conferring resistance to RIF and INH (i.e., MDR).

In this study, the frequency of mutation at Codon S531L occurred more in two of the three MDR strains and in one of the six RIF mono-resistant strains. However, higher frequency of mutation at Codon S531L was reported from India and South Africa in MDR and RIF mono-resistant strains [16,20].

In the GenoType MTBDR*plus* assay, resistance to INH is detected by probes of the *katG* and *inhA* genes. The higher frequency of resistance to INH occurred due to mutation of the *katG* gene, whereas lower frequency of resistance was caused by the mutations in the promoter region of the *inhA* gene [23]. Of the 25 INH-resistant isolates, *katG* mutation occurred in 88% (22/25) of the isolates. In all of these 22 isolates, specific mutations were found at Codon S315T1 of the *katG* gene, which was also reported by other studies conducted in northwest Ethiopia [15] and India [19]. Some studies reported lower frequencies of mutation in the *katG* gene at Codon S315T1 from Uganda, France, and South Africa [17,18,20].

Mutations in the *inhA* gene occurred in only three of the 25 INH-resistant isolates, which is similar to the frequency of *inhA* mutation reported from the northwest part of Ethiopia [15]. Compared with these findings, a study from North India reported the occurrence of low frequency of INH-resistance mutation in the *inhA* gene [19]. In contrast to these findings, higher *inhA* gene mutation was reported from Tunisia [24] and Canada [25]. Specific *inhA* mutations were found in two of the three INH-resistant isolates, which had mutation in Codon C15T, but in the remaining one isolate, the *inhA* WT2 gene was missing without the presence of a specific mutation band. In this study, four of the phenotypically defined INH-resistant strains had no mutations in the *katG* and *inhA* genes. This could suggest that there may be other mutations in other codons of the *katG* and *inhA* genes or the presence of some unidentified mutations in other genomic regions such as *ahpC, kasA,* and *furA*.

In conclusion, the findings of this study revealed that the GenoType MTBDR*plus* assay has high sensitivities and specificities for detecting RIF- and INH-resistant, and MDR *M. tuberculosis* isolates, suggesting the potential role of this assay for the control of TB in Ethiopia and other countries.

Conflicts of interest

The authors declare that they have no conflicts of interests.

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