MAJOR ARTICLE

Extensively Drug-Resistant Tuberculosis: 2 Years of Surveillance in Iran

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Background. Extensively drug-resistant (XDR) tuberculosis (TB) is a cause of concern, because it renders patients untreatable with available drugs. In this study, we documented the existence and transmission of XDR TB among patients with multidrug-resistant TB. These patients were referred to the National Research Institute of Tuberculosis and Lung Diseases (Tehran, Iran) for treatment and diagnosis from 2003 to 2005.

Methods. The sputum specimens from a total of 2030 patients with TB were digested, examined microscopically for acid-fast bacilli, and inoculated into Löwenstein-Jensen slants by standard procedures. Testing of susceptibility to first-line drugs was performed for 1284 *Mycobacterium tuberculosis* isolates. Subsequently, the strains that were identified as multidrug-resistant *M. tuberculosis* (113 isolates) were subjected to susceptibility testing for second-line drugs. Spoligotyping and restriction fragment–length polymorphism were performed for strains that were identified as XDR *M. tuberculosis.*

Results. A total of 12 (10.9%) of 113 multidrug-resistant *M. tuberculosis* strains were resistant to all 8 second-line drugs tested and, therefore, were denoted as XDR *M. tuberculosis.* Retrospective analysis of the cases of XDR TB showed that all of them belonged to 1 of 2 epidemiological clusters, either a single-family cluster (4 cases) or a cluster of close contacts (8 cases). The strains were identified as belonging to the *M. tuberculosis* superfamilies Haarlem 1 and East African Indian 3.

Conclusions. The emergence of XDR TB cases in Iran highlights the need to reinforce the Iranian TB policy with regard to control and detection strategies.

Tuberculosis (TB) represents a serious public health problem in Iran. According to the World Health Organization, the estimated incidence rate of TB is 28 cases per 100,000 population [1]. The TB problem has become more severe because of an increase in multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains that are resistant to isoniazid and rifampicin. According to a nationwide survey conducted in 1999, among all *M. tuberculosis* isolates tested for drug susceptibility, 10.9% were resistant to $\geq$1 anti-TB drug, and 6.7% were resistant to both isoniazid and rifampin (i.e., were MDR strains of *M. tuberculosis*) [2]. It is generally accepted that drug-resistant or MDR TB develops through the selection of drug-resistant strains in patients who are receiving suboptimal drug regimens or who fail to complete chemotherapy with the appropriate combination of drugs [3, 4]. In addition, the spread of resistant bacteria from such patients is another factor that makes the epidemiological characteristics of MDR TB even more severe. Indeed, isoniazid and rifampicin are the 2 most important drugs in the treatment of TB, and resistance to these drugs, and particularly to rifampicin, represents a major risk factor for treatment failure with the recommended standard TB therapy. Nevertheless, there has been encouraging evidence in recent years that patients with MDR TB can be cured with appropriate combinations of second-line drugs [4–7]. Mirsaedi et al. [8] showed that 76% of Iranian patients with MDR TB responded well to the second-line treatment within 18.5 months after treatment initiation. However, these drugs are less effective, more expensive [6, 7], and demand longer treatment periods. Therefore, the best way to stop MDR

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TB is to detect and treat drug-susceptible or drug-resistant TB before it evolves into MDR TB and before it spreads. For this reason, it is essential to perform drug-susceptibility testing on the first-line drugs, to identify patients with drug-resistant and MDR TB, and, for patients receiving the relevant second-line agents, to optimize the treatment of drug-resistant disease. Therefore, we performed drug-susceptibility testing of 113 MDR *M. tuberculosis* strains isolated from 113 patients for the second-line drugs that are commonly used in Iranian drug regimens for the treatment of MDR TB cases (amikacin, kanamycin, capreomycin, ethionamide, para-aminosalicylic acid, cycloserine, and ofloxacin). Our study identified a considerable number of MDR *M. tuberculosis* strains that were resistant to most or all of the tested second-line drugs. These strains are referred to as extensively drug-resistant (XDR) *M. tuberculosis* strains according to the Centers for Disease Control and Prevention and the World Health Organization. Considering the severity of disease associated with the presence of such XDR *M. tuberculosis* strains, we tried to increase the understanding of the relationship between the XDR TB cases in Iran using both classical and molecular epidemiological techniques. To our knowledge, this is the first report that describes the transmission of XDR *M. tuberculosis* strains among patients with secondary cases of TB.

MATERIALS AND METHODS

Setting. The National Research Institute of Tuberculosis and Lung Diseases, which acts as the reference unit for the National Tuberculosis Program, is the only center for diagnosis and treatment of patients with MDR TB in Iran. Patients from throughout Iran who have clinically confirmed cases of TB are referred to the National Research Institute of Tuberculosis and Lung Diseases for culture and susceptibility testing. Strains that were identified as MDR *M. tuberculosis* (and which were obtained from patients with complete follow-up information) were selected for further investigation. We performed a cross-sectional study of the data.

Data collection. Clinical and epidemiological information was collected from patients with confirmed cases of TB by trained technicians using standard questionnaires. Information was obtained on sex, age, contact with another patient with TB, duration of contact, previous history of TB, present address, and associated medical data (such as presence of HIV infection, tuberculin skin test results, and chest radiograph findings). The patients with similar or highly similar IS6110-based restriction fragment–length polymorphism (RFLP) fingerprint patterns were interviewed together. The study was approved by the institutional review board at the National Research Institute of Tuberculosis and Lung Diseases in Tehran, Iran.

Patients with MDR TB. Inclusion criteria for patients with MDR TB were a history of at least 1 previous period of TB treatment under the center's direct observation (with at least 6 months of documentation), 2 positive sputum smear test results, and a positive sputum culture result. Patients were also required to have drug-susceptibility test results that showed resistance to isoniazid and rifampin, as well as chest radiograph findings and clinical symptoms that were compatible with pulmonary TB.

Treatment. According to the National Tuberculosis Program, new cases of TB are treated with 4 first-line drugs: isoniazid (5 mg/kg), rifampicin (10 mg/kg), pyrazinamide (25 mg/kg), and ethambutol (15 mg/kg). If the smear examination has positive findings after 5 months of treatment, the treatment is considered to have failed, and patients are hospitalized with suspected MDR TB. Chemotherapy for each patient is administered on the basis of his or her drug treatment history. Drug regimens mainly comprised a combination of first- and second-line drugs. An aminoglycoside (usually amikacin administered at a dose of 15 mg/kg) was included in the treatment program for all patients unless it was contraindicated. All patients received ofloxacin (600–800 mg per day), and cycloserine, if prescribed, was given at a dosage of 500 mg per day. Because of the lack of ethionamide, isoniazid, administered at a dosage of 15 mg/kg, was added to all regimens for the treatment of MDR TB. The treatment period continued for at least 18 months after the first sputum culture that showed no growth or for at least 24 months when a second-line drug was used. After discharge from the hospital, follow-up evaluations included a sputum smear and culture every month and a chest radiograph every 3 months [8].

Bacterial strains. The primary isolation and culture of *Mycobacterium* isolates, after NaOH-N-acetylcysteine (Becton Dickinson Diagnostic Systems) treatment, was done in accordance with standard solid-culture procedures [10]. All isolates were identified as *M. tuberculosis* complex with use of standard biochemical tests, including production of niacin, catalase activity, and nitrate reduction, as well as the registration of pigment production and growth rate. All the drugs were purchased from Sigma Chemical (St. Louis, MO). Drug-susceptibility testing against isoniazid, rifampicin, streptomycin, and ethambutol was performed by the proportional method on Löwenstein-Jensen media at a concentration of 0.2, 40, 4.0, and 2.0 μg/mL, respectively. Susceptibility to pyrazinamide (900 and 1200 μg/mL) was tested using a 2-phase medium where the strain was reported to be resistant to pyrazinamide if, on day 21, the proportion of drug-resistant colonies was higher than the defined critical proportion. Drug-susceptibility testing against second-line drugs (kanamycin, amikacin, capreomycin, ciprofloxacin, cycloserine, ethionamide, and para-aminosalicylic acid) was performed using 2 critical proportions of 1% and 10% [11].

DNA fingerprinting. Extraction of bacterial DNA and

DNA fingerprinting with RFLP using IS6110 as a probe was performed with use of standard protocols [12]. In brief, for isolation of genomic DNA, *M. tuberculosis* strains were grown on Löwenstein-Jensen slants for 3–5 weeks. All bacterial cells from 1 slant were transferred in 400 μL of TE buffer (0.01 M Tris-HCL and 0.001 M EDTA; pH, 8), and the solution was heated at 80°C for 20 min to kill the bacteria. A total of 50 μL of lysozyme (10 mg/mL) was added, and the tube incubated for 1 h at 37°C. A total of 70 μL of sodium dodecyl sulfate (10%) and 6 μL of proteinase K (10 mg/mL) was added, and the mixture was incubated for 10 min at 65°C. A 100-μL volume of 5 M NaCL and of an N-cetyl N, N, N–trimethylammonium bromide–NaCL solution (4.1 g of NaCL and 10 g of trimethylammonium bromide per 100 mL) were added. The cups were vortexed and incubated for 10 min at 65°C. An equal volume of chloroform-isoamylalcohol (24:1) was added, the mixture was centrifuged for 5 min at 12,000 *g*, and the aqueous supernatant was carefully transferred to a fresh tube. The total DNA was precipitated using isopropanol and was redissolved in an appropriate volume of double-distilled water. The PVUII-digested DNA was separated using horizontal 1% agarose gels and was capillary-blotted onto a nylon membrane. Hybridization of the DNA was performed with a 254–base pair internal PCR fragment of IS6110 as a probe using the ECL system (Amersham). PVUII-digested total DNA of *M. tuberculosis* reference strain 14323 was used in each Southern blot experiment as an external size standard [12].

Spoligotyping. Spoligotyping was performed as described by Kamerbeek et al. [13] with a commercially available kit (Isogen Bioscience). In brief, the direct repeat region was amplified by PCR using primers derived from the direct repeat sequence. The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequence of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 by reverse line blotting.

Computer-assisted analyses of fingerprints. The autoradiograph of the IS6110 RFLP fingerprint was scanned with Snap Scan 1236 scanner (AGFA). Bionumerics software, version 2.5

Table 1. Demographic and clinical information for patients with multidrug-resistant *Mycobacterium tuberculosis* infection, by nationality.

Variable	Iranian patients (n = 41)	Afghani patients (n = 72)	All patients (n = 113)	P
Percentage of all patients	36.3	63.7	100	
Sex				
Female	19 (46.3)	29 (40.3)	48 (42.5)	NS
Male	22 (53.7)	43 (59.7)	65 (57.5)	
Age, mean years ± SD	43.1 ± 17.6	31.8 ± 16.3		
Injection drug user				
No	33 (80.5)	NA	33 (29.2)	<.05
Yes	8 (19.5)	NA	8 (7.1)	
HIV infection status				
Negative	34 (82.9)	NA	34 (30.1)	<.05
Positive	7 (17.1)	NA	7 (6.2)	
Family and/or close contact with TB	16 (39.0)	29 (40.83)	45 (39.8)	NS
Previous history of TB	20 (48.8)	35 (48.6)	55 (48.7)	
Newly positive smear results	5 (12.2)	8 (11.1)	13 (11.5)	
PPD				
Positive	30 (73.2)	59 (81.9)	89 (78.8)	NS
Negative	11 (26.8)	13 (18.1)	24 (21.2)	
Duration of contact with individual with TB				
<6 months	7 (17.1)	13 (18.1)	20 (17.7)	NS
6 months	12 (29.3)	14 (19.4)	26 (23.0)	
>6 months	22 (53.7)	45 (62.5)	67 (59.3)	
Radiological finding				
Cavitary lesions	9 (22.0)	18 (25.0)	27 (23.9)	NS
Bilateral pulmonary involvement with cavitary lesions	17 (41.5)	35 (48.6)	54 (47.8)	
Bilateral pulmonary involvement	11 (26.8)	12 (16.7)	21 (18.6)	
Noncavitary nonbilateral pulmonary involvement	4 (9.8)	7 (9.7)	11 (9.7)	

NOTE. Data are no. (%) of patients, unless otherwise indicated. NS, not significant; TB, tuberculosis.

(Applied Math) was used to analyze the molecular patterns generated by the IS6110 RFLP. The similarity matrices were constructed using the Jacquard index with a linear error tolerance of 1%–3% proportional to the size of the bands. The dendograms were generated by the hierarchic unweighted pair-group method analyses. Strains were classified as a cluster if they had banding patterns that were 100% similar.

Statistical analysis. The continuous variables were expressed as group means (± SDs). The primary variable was the drug-susceptibility test result. Secondary variables included sex, age, whether the patient was a drug abuser, HIV status, whether a family member or close contact had TB, the pattern of drug resistance, the result of a purified protein derivative test, the duration of contact with a TB-positive individual, and the *M. tuberculosis* superfamily. A χ^2 test, Fisher's exact test, Student's *t* test, and the Mann-Whitney *U* test were used, as appropriate. All *P* values were 2-tailed values. A *P* value of <.05 was considered to be statistically significant. Findings were analyzed using SAS software, version 9.01 (SAS).

RESULTS

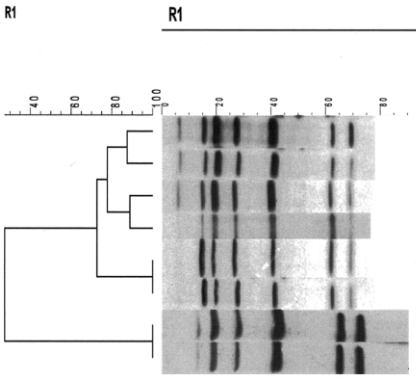
Study population. From January 2003 to January 2005, a total of 2030 sputum specimens were collected from patients with clinically confirmed cases of TB. Of these specimens, only 1284 (63.3%) had a positive culture result. The remaining specimens were excluded, because they were either culture negative (611 specimens; 30.1%) or culture contaminated (135 specimens; 6.7%). The results of susceptibility testing showed that 695 (54.1%) of the specimens with a positive culture result had isolates that were susceptible to all 4 drugs tested. Of the 1284 specimens with a positive culture result, 150 (11.7%) had isolates with multidrug resistance, and 439 (34.2%) had isolates with mixed resistance. Of 150 patients with MDR TB, 113 (75.3%) who had complete follow-up information were selected for further analysis. The demographic characteristics of these patients are shown in table 1. The majority of them (including both Afghani and Iranian patients) had a previous history of TB (55 [48.7%] of 113) or had close contact with an individual

Table 2. Demographic and clinical characteristics for patients with extensively drug-resistant *Mycobacterium tuberculosis* infection.

Variable	Patients with extensively drug-resistant *M. tuberculosis* infection with the specified characteristic[a]	Percentage of all patients with extensively drug-resistant *M. tuberculosis* infection (*n* = 12)	Percentage of all patients with multidrug-resistant *M. tuberculosis* infection (*n* = 113)
Sex			
Female	1	8.3	0.9
Male	11	91.7	9.7
Age, mean years ± SD	28.4 ± 13	…	…
Injection drug user			
Yes	8	66.7	7.1
No	4	33.3	3.5
HIV infection status			
Negative	9	75.0	8.0
Positive	3	25.0	2.7
Family and/or close contact with TB	12	100.0	10.6
Previous history of TB	0	0.0	0.0
Newly positive smear results	12	100.0	10.6
PPD			
Positive	12	100.0	10.6
Negative	0	0.0	0.0
Duration of contact with individual with TB			
<6 months	5	41.7	4.4
6 months	3	25.0	2.7
>6 months	4	33.3	3.5
Radiological finding			
Cavitary lesions	3	25	2.7
Bilateral pulmonary involvement with cavitary lesions	8	66.7	7.1
Bilateral pulmonary involvement	1	8.3	0.9
Noncavitary nonbilateral pulmonary involvement	0	0.0	0.0

NOTE. All patients with extensively drug-resistant *M. tuberculosis* infection were of Iranian nationality. TB, tuberculosis.

[a] Data are no. of patients, unless otherwise indicated.



Isolate	Date of culture	Duration of Contact	Classical Cluster confirmation	Spoligotype
I.a	Feb 2002	9 months	Sharing room with MDR TB– and HIV-positive patients (index patient)	
I.b	Jun 2002	4 months	Brother of index patient	
I.c	Jun 2002	4 months	Brother of index patient	
I.d	Dec 2002	6 months	Sharing room with index patient	Haarlem 1
I.e	Sep 2002	3 months	Sharing room with index patient	
I.f.	Jul 2002	7 months	Sharing room with index patient	
I.g	Aug 2002	8 months	Sharing room with index patient	
I.h	Nov 2002	13 months	Sharing room with index patient	

Figure 1. Data for an outbreak of extensively drug-resistant *Mycobacterium tuberculosis* that occurred among a family and community (cluster I). The index patient (I.a) acquired tuberculosis from an individual with multidrug-resistant tuberculosis and transmitted the disease to his family and friends.

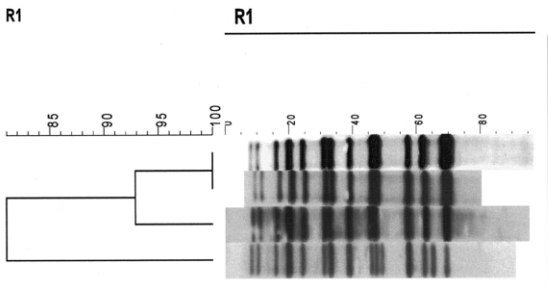
with a known case of MDR TB (45 [39.8%] of 113). Reviewing the questionnaires of the Afghani patients revealed that most of them had been living in Iran for 15–20 years. However, we could not determine whether they had been infected before or after migration to Iran. Second-line drug susceptibility testing of these strains revealed that 12 (10.6%) of 113 strains were resistant to all 8 second-line drugs tested. Notably, they were all isolates obtained from patients with newly positive smear results who had acquired TB through transmission (table 2). The duration of contact ranged from a minimum of 3 months to a maximum of 13 months. Each of these patients continued to have positive smear results after 24 months of chemotherapy.

RFLP of patients with XDR TB. On the basis of the IS6110 RFLP banding pattern, we identified 2 clusters (cluster I and cluster II) of XDR TB cases. Cluster I contained 8 patients and involved an outbreak in both a family and a community. The first patient in this cluster, denoted as patient I.a, was a drug abuser and shared a room for 9 months with an HIV-positive patient with MDR TB. Four months later, 2 of his family members (patients I.b and I.c) developed active disease, but their HIV test results were negative. Within 1 year, 5 more patients (patients I.d, I.e, I.f, I.g, and I.h) were referred to the hospital; they could identify patient I.a as a close contact. These patients had been sharing the same room, and all of them were injection drug users. Two of the patients were HIV positive (figure 1). Similarly, the first patient in the second cluster (patient II.a) acquired TB from his brother, who had had smear-positive MDR TB for >1 year. The drug-susceptibility test results for the isolate from patient II.a showed resistance to all the second-line drugs tested. In less than 1 year, patient II.a transmitted the disease to his family members (patients II.b, II.c, and II.d) (figure 2).

Distribution of the 12 XDR TB spoligotypes. When the spoligotypes from the isolated XDR *M. tuberculosis* strains were compared with earlier published spoligotypes, our isolates could be identified as members of the superfamilies Haarlem 1 (8 isolates) and East African Indian 3 (4 isolates).

DISCUSSION

In the present study, we describe 2 clusters of XDR TB. The XDR *M. tuberculosis* isolates were resistant to isoniazid and



Isolate	Date of culture	Duration of contact	Classical cluster confirmation	Treatment
II.a	Nov 2000	6 months	Father (index patient)	Treatment failure, persistence of positive smear or culture results despite treatment for at least 18 or 24 months
II.b	Mar 2002	4 months	Cousin of index patient	
II.c	Sep 2002	6 months	Daughter of index patient	
II.d	Jul 2002	3 months	Brother of index patient	

Figure 2. Data for an outbreak of extensively drug-resistant *Mycobacterium tuberculosis* that occurred among a family (cluster II). The period during which transmission occurred was 3–6 months in duration. On the basis of spoligotyping results, these strains were determined to belong to the East African Indian 3 *M. tuberculosis* superfamily.

rifampin and to at least 3 of the 6 main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid) [9]. They not only constitute a deadly threat to the affected patients with TB but also hamper the TB-control program. If these extensively resistant pathogens are allowed to develop and spread in society, they will constitute a significant public health problem [14, 15]. Based on standard protocols, if a patient has an isolate that is resistant to all but 2 or 3 relatively weak drugs, they should undergo surgery [16]. However, in our study, surgery was not applicable, because the patients had large cavities and very poor lung function. Indeed, they were receiving the combination of first- and second-line drugs without any improvement. This problematic situation illustrates an urgent need to find an effective medicine for treating such complicated cases. Fortunately, at present, the number of XDR TB cases in our country is still very low, but the higher rate of TB cases, including MDR TB cases, in neighboring countries like Afghanistan, Pakistan, and the countries of the former Soviet Union, underlines the probability that similar cases also exist in these countries. Recently, a population-based study of drug susceptibility among isolates from patients with TB showed that 4%, 19%, and 15% of MDR TB cases in the United States (for 1993–2004), Latvia (2000–2002), and South Korea (2004), respectively, were XDR TB [9]. However, in a study involving 69 MDR *M. tuberculosis* isolates from the Samara region of Russia [17], only a few strains showed resistance to prothionamide (1 of 69 strains), ciprofloxacin (3 of 69), and amikacin (5 of 69). In another recent study from Archangels in northwest Russia [18], only 1 of 77 strains from patients with MDR TB was resistant to ethionamide, kanamycin, and ofloxacin, and, thus, only 1 case could be classified as XDR TB [18]. Overall, there have been only a few reports of XDR TB published [4, 9, 14, 18, 19], and the drugs included, as well as the drug-susceptibility testing techniques used, vary between studies, making the direct comparison of results difficult. In a study from Hong Kong [19], strains with an extensive resistance to second-line drugs were only found in isolates from patients with MDR TB. XDR TB (here defined as simultaneous resistance to ethinoamide, amikacin, ofloxacin, and cycloserine) was seen in 9 (12%) of 75 patients, a rate that is similar to our findings in Iran. In an Ethiopian study [20], no XDR TB was detected among 13 patients with MDR TB. All 13 isolates were susceptible to ethinoamide, ciprofloxacin, and amikacin. Thus, the spectrum of resistance reflects the drugs that the patient has used and the way in which the therapy was controlled. The worst problems are expected in areas like Iran, in which many different second-line agents have been used in a poorly controlled manner. In this study, the strains identified as being associated with XDR TB were fully capable of being transmitted and causing active disease in individuals with secondary cases. This finding is very important, because it clearly underlines the urgent need to reinforce the Iranian TB-control policy, with special regard to the prompt and reliable laboratory detection of drug-resistant TB, as well as the need for efficient infection-control measures to stop or strongly limit the spread of drug-resistant *M. tuberculosis.* The results of this study also show that the most frequently occurring superfamilies (or clades) among patients with XDR TB are Haarlem 1 (8 cases; 66.7%), and East African Indian 3 (4 cases; 33.3%). The Haarlem 1 *M. tuberculosis* family was first isolated from a patient living in Haarlem, The Netherlands [21]. Today, the widespread distribution of Haarlem 1*M. tuberculosis* in different geographical regions of the world, including Asia, Europe, and Africa, has been documented [21, 22]. In addition, outbreaks due to Haarlem 1 *M. tuberculosis* have been reported in the Czech Republic [23] and Tunisia [24]. Another identified superfamily was the East African Indian 3 superfamily, which is characterized by the presence of spacer 33 and the absence of spacer 34 [21]. In Europe and Australia, the East African Indian 3 superfamily has been regularly linked with immigrants from central Asia and the Middle East. We have already demonstrated [25] that these 2 superfamilies account for the majority of *M. tuberculosis* isolates from Iranian patients with TB. However, from an epidemiological point of view, it would be necessary to perform a more extensive surveillance study to determine whether these strains are responsible for XDR TB transmission within Iran. Last but not least, our data strongly show the need for an increased capacity with respect to high-quality susceptibility testing of first-line drugs to detect MDR TB and show the need for proper second-line drug-susceptibility testing to enable an optimized treatment of drug-resistant TB. In addition, the need for an effective infection-control program is obvious.

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Potential conflicts of interest. All authors: no conflicts.

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