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Research Article

Diagnostic Accuracy of Direct Smear Microscopy Using Culture as a Gold Standard, among Pulmonary Tuberculosis Patients in Anambra State, Nigeria

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Abstract

Background: Nigeria has one of the highest burdens for TB, TB/HIV and multi-drug resistant TB in the world. Accurate and prompt identification of *Mycobacterium tuberculosis* for treatment of tuberculosis disease is essential to reducing the global burden. This study aimed at determining the sensitivity, specificity and other measures of diagnostic accuracy of direct smear microscopy using culture as the reference standard.

Methods: This was a cross-sectional study of presumptive tuberculosis patients who presented to selected hospitals in Anambra state, South-eastern Nigeria. A total of 550 sputum samples were assessed for *Mycobacterium tuberculosis* using direct smear microscopy by Ziehl-Nelson staining technique and culture by Lowenstein-Jensen solid medium, as diagnostic reference standard.

Results: Tubercle bacilli was detected by direct smear microscopy in 160 sputum samples (29.1%) whereas sputum culture was positive for *Mycobacterium tuberculosis* in 180 samples (32.7%). Out of 180 sputum culture positive TB patients, 34 patients (18.9%) were positive for human immunodeficiency virus in contrast to only 20 (12.5%) detected by direct smear microscopy. Diagnostic accuracy of direct smear microscopy was 82.2% (95% CL 77.8% - 87.5%). Specificity, 78.9% (95% CL 69.8% - 84.9%) was low compared to culture; sensitivity 88.9%, false positive rate 21.1% and false negative rate 11.1%.

Conclusion: To improve accuracy of TB case detection, culture should be used routinely as a backup to enhance the specificity of direct smear microscopy especially in HIV-positive patients.

Keywords: *Tuberculosis; Mycobacterium tuberculosis; Direct Smear Microscopy; Sputum Culture; Anambra State*

Abbreviations

TB: Tuberculosis; MTB: *Mycobacterium tuberculosis*; NTM: Non-Tuberculosis Mycobacteria; HIV: Human Immune Deficiency Virus; DSM: Direct Sputum Smear Microscopy; AFB: Acid Fast Bacilli; ZN: Ziehl- Nielsen; LJ: Lowenstein Jensen; DOT: Direct Observe Treatment; SOP: Standard Operating Procedure; NAOH: Sodium Hydroxide; (-): Negative; (+): Positive; SPSS: Statistical Package for Social Sciences;

CL: Confidence Interval; TP: True Positive; TN: True Negative; FP: False Positive; FN: False Negative; PPN: Positive Predictive Value; NPV: Negative Predictive Value; LR^+: Positive Likelihood Ratio; LR: Negative Likelihood Ratio; DOR: Diagnostic Odd Ratio; X^2: Chi-Square; ROC: Receiver Operating Characteristics; NAUTH: Nnamdi Azikiwe University Teaching Hospital; COOUTH: Chukwuemeka Odumegwu Ojukwu University Teaching Hospital; GHO: General Hospital Onitsha; OHI: Our Lady of Lourdes Hospital Ihiala

Introduction

Tuberculosis is a curable and preventable communicable disease caused by *Mycobacterium tuberculosis (MTB)*. It typically affects the pulmonary system causing inflammation and lesions of lung tissue. Transmission is by respiratory route through inhalation of the organism in the droplets of the bronchial secretions of infected persons [1]. *M. tuberculosis*, a bacterium classified as a gram positive rod, is characterized by resistance to acid-alcohol decolourization observed through Ziehl-Nielsen dyeing [2]. The major challenge of *M. tuberculosis* infection and pathogenesis is latency; in about 20% of all cases, the bacilli remain latent in the affected lymph nodes and there may not be clinical, radiological and bacteriological evidence of TB [3]. According to World Health Organization, persons at risk for developing tuberculosis disease is about 5 - 10% of infected persons who do not receive treatment for latent TB infection and persons whose immune systems are weak especially those with diabetes mellitus, kidney diseases, organ transplants, head and neck cancers and HIV infection. The risk among HIV/TB co-infected persons is 5-15% within a year [4].

Globally in 2019, 9,960,000 people were infected with *M. tuberculosis*. There were 1.2 million deaths among HIV negative people, 208,000 deaths from TB among HIV-positive people and treatment coverage of 71%. A combination of under diagnosis and underreporting of detected cases contributes to a wide gap between numbers of incident TB cases and those diagnosed and notified [5,6]. Nigeria has one of the highest burdens for TB, TB/HIV and multi-drug resistant TB in the world. The country also accounts for 11% of the global gap between the number of incident TB cases and those diagnosed and notified [6]. Out of 440,000 estimated TB cases in 2019, only 120,266 were notified and only 27% of this number received treatment [5]. Also TB preventive treatment to HIV-positive people declined from 62% in 2018 to 50% in 2019 [5,7], it however increased from 19% to 33% in children younger than 5 years who are household contacts of bacteriologically-confirmed TB cases [5]. There are regional differences in TB case detection and TB/HIV co-infection rates reported in Nigeria [8,9].

To end *M. tuberculosis* transmission, active case finding with affordable and sustainable diagnostic techniques and effective treatment are needed. Diagnosis of *M. tuberculosis* from clinical specimens using culture method is a reference standard for confirmation of TB infection and drug susceptibility testing [10] but culture methods are not routinely implemented for TB diagnosis in Nigeria possibly due to its high cost and infrastructural requirements [11]. The Xpert MTB/RIF® test is recommended as the primary TB diagnostic tool by the National Tuberculosis and Leprosy Control Programme. However, compared with the reference standard of culture, Xpert MTB/RIF® has suboptimal specificity and sensitivity (especially in individuals with smear-negative TB and people living with HIV) [6]. Furthermore, Nigeria has a low coverage of the Xpert MTB/RIF® technology for TB diagnosis. Only 6.3% of primary health care facilities in the country had access to the test in 2017 [12] leaving them to resort to diagnosing of TB by identification of acid-fast bacilli (AFB) on direct sputum smear microscopy.

A challenging problem globally is establishing bacteriological confirmation of tuberculosis in HIV infected patients due to low density of acid-fast bacilli in their sputum especially in resource-limited countries in Africa where HIV cases are endemic. Research has shown that the sensitivity of direct sputum smear microscopy for identification of cases of TB is within the range of 60 - 80% under optimal conditions compared to culture but it varies in some settings as low as 22 - 43% [13,14]. This sensitivity reduces to 20% in HIV- and paediatric-associated TB [15,16]. Previous work in Brazil on the accuracy of direct sputum smear microscopy reported a sensitivity of 36% and specificity of 100% in HIV-positive patients [17].

Objective of the Study

The objective of this study therefore, was to determine the diagnostic accuracy of direct sputum smear microscopy for diagnosis of TB among HIV co-infected patients in Anambra state, South-eastern Nigeria.

Materials and Methods

Study area

The study was done at four selected hospitals in Anambra state, South-eastern Nigeria; Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi (a tertiary care public hospital), General Hospital, Onitsha (a secondary care public hospital), Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, (COOUTH) Awka (a tertiary care public hospital) and Our Lady of Lourdes Hospital (OHI), Ihiala (a private, secondary care hospital).

Study population

This was a cross sectional laboratory based study, which was carried out with sputum samples obtained from patients who had presented to TB directly observed treatment (DOT) clinics in four selected hospitals in Anambra state, Nigeria from 2010 - 2012.

Inclusion criteria: They were male and female presumptive TB cases of all ages who provided written informed consent to HIV counselling and testing for the study. All eligible presumptive TB cases who presented at the DOT clinics during the study period were examined. Patient demographic data were obtained by questionnaire.

Exclusion criteria: Patients who refused to undergo HIV testing.

Ethical approval

Ethical approval was obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria (reference number: NAUTH/CS/66/VOL.3/35). Information on the purpose of the study was provided to all the participants. They were assured of their voluntary participation, the confidentiality of their data and the freedom to withdraw from the study at any time. All individual participants provided written informed consent.

Laboratory diagnosis

- **Specimen collection:** On-the-spot and early morning sputum samples of 550 presumptive TB suspects were collected in sterile screw-capped containers and maintained at temperature of 4^0C - 8^0C. The blood samples of all patients (5 mls) were collected in a dry sterile screw capped containers for HIV screening.

- **Diagnostic procedure:** Direct smear microscopy (DSM) and culture were two methods used. The sputum sample of each patient was processed within 24 hours of collection by DSM using Ziehl-Neelsen (ZN) staining technique. Each sputum smear was examined and quantified using standard operating procedures [18]. Sputum samples were sent to Zanklin Medical Centre Abuja in cold ice packs where cultures on Lowenstein-Jensen (LJ) solid medium were done. There, sputum samples were decontaminated by centrifugation at 3000g for 15 minutes. Sediments were re-suspended in 2 ml phosphate buffer at pH 6.4 and 2 drops of the sediments were inoculated on slopes of already prepared LJ medium. The inoculated LJ slopes were incubated at 37^0C and examined on 4th day for contamination and weekly for 6 - 8 weeks for growth of *M. tuberculosis* [18]. Patient HIV screening was done using Stat-Pak and Determine according to manufacturer descriptions.

Quality assurance

Standard operating procedure were observed (SOP). Positive (+) and negative (–) control slides were used for internal quality control procedure and for staining reagents. Other quality control measures were done on the culture using known positive strain of H37RV *M. tuberculosis* and negative strain, including monitoring the performance of indicators such as contamination rate.

Statistical analysis

Data was analysed using Statistical Package for the Social Sciences (SPSS) version 21 (International Business Machines, Chicago, USA). A two-by-two contingency table was used for comparison of DSM with culture. Frequencies, proportions and 95% confidence intervals (CI) determined for true positives (TP), true negatives (TN), false positives (FP), as well as false negatives (FN) and these were used to inputted into standard formulae to derive the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR-), diagnostic odds ratio (DOR) and accuracy. Categorical variables were compared using Chi-square test of significance. Receiver operating characteristic (ROC) curve analysis was also conducted and the area under curve determined. Statistical significance was set at 95% confidence level with p value < 0.05.

Results

Sputum samples of 550 presumptive TB patients were examined for acid-fast bacilli (AFB) using direct sputum smear microscopy and *Mycobacterium tuberculosis* were identified on LJ solid culture medium. Direct sputum smear microscopy yielded 160 (29.1%) positive AFB, 10 (1.8%) had non-tuberculosis mycobacteria (NTM) and 380 (69.1%) negative AFB while culture yielded 180 (32.7%) positive *M. tuberculosis*, 20 (3.6%) NTM, 38 (6.9%) were contaminants and 312 (56.7%) were culture negative.

The study samples comprised 318 males (57.8%) and 232 females (42.2%) with an age range of 7 to 81 years. Out of the 180 culture-positive cases, 109 (60.5%) were males and 71 (39.4%) females. (P-value = 0.543). Majority (65%) were within 21 - 40 years of age group. Children and young people, 20 years and younger and the elderly, 61 years and older each had the lowest TB prevalence rates of 7.8% (Table 1).

Characteristic	Sputum culture				χ^2	P-value
	Positive (n = 180)		Negative (n = 370)			
	n	%	n	%		
Age (years)					20.76	0.000118
≤ 20	14	7.8	31	8.4		
21 - 40	117	65.0	167	45.1		
41 - 60	35	19.4	130	35.1		
≥ 61	14	7.8	42	11.4		
Sex					2.15	0.543
Male	109	60.6	209	56.5		
Female	71	39.4	161	43.5		

Table 1: *Age and sex distribution of sputum culture results for presumptive tuberculosis cases in Anambra state, south-eastern Nigeria. χ^2: Chi-square.*

Out of 550 patients examined, 130 (23.6%) were HIV-positive and 420 (76.4%) HIV-negative. Of 180 TB culture-positive patients, 34 (18.9%) were HIV-positive while 146 (81.1%) were HIV-negative. In contrast, using DSM analysis, out of the 160 TB positive patients, 20 (12.5%) were HIV-positive and 140 (87.5%) were HIV-negative as shown in table 2.

HIV Status	DSM		Sputum culture	
	Positive%	Negative%	Positive%	Negative%
Positive (n = 130)	20 (12.5)	110 (28.2)	34 (18.9)	96 (25.9)
Negative (n = 420)	140 (87.5)	280 (71.8)	146 (81.1)	274 (74.1)
Total (n = 550)	160 (29.1)	390 (70.9)	180 (32.7)	370 (67.3)

Table 2: *Distribution of direct sputum smear microscopy and sputum culture, categorised by HIV status, among TB cases.*

Among the TB/ HIV-positive patients, 20 (15.4%) were both culture positive/DSM positive TB/HIV cases; 14 (10.8%) were culture positive TB/HIV/DSM positive TB/HIV negative cases and 4 (3.1%) were culture negative TB/HIV cases/DSM positive TB/HIV cases. Also, among the TB positive/HIV-negative cases, 120 (28.6%) were culture positive TB/DSM positive TB/HIV negative cases; 20 (4.8%) were culture positive TB/DSM negative TB/HIV negative cases and 6 (1.4%) were culture negative TB/DSM positive TB/HIV negative, as shown in figure 1.

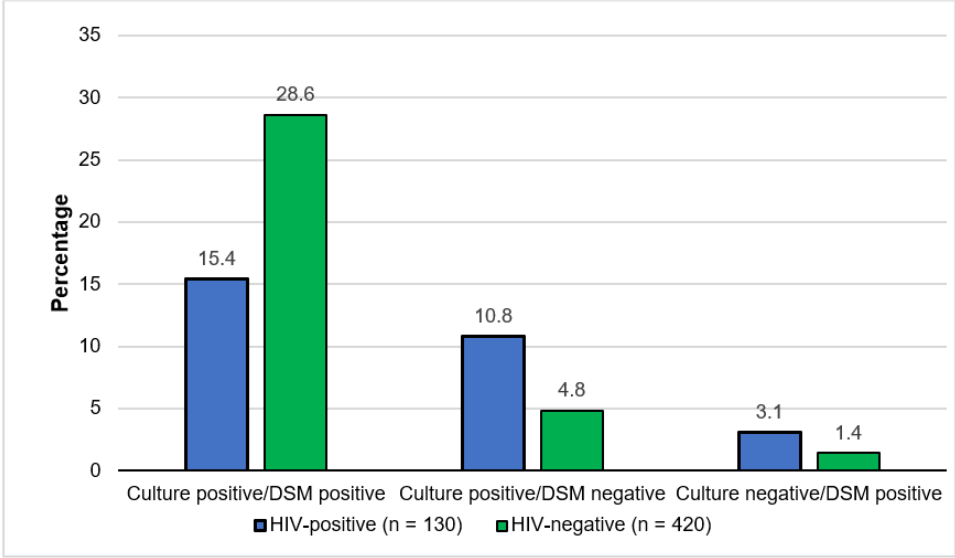

Figure 1: *Comparing the proportions of direct smear microscopy and sputum culture results by HIV status, among tuberculosis patients.*

A cross tabulation comparing the diagnostic performance of DSM with the reference standard culture (Table 3). The difference in diagnostic performance of DSM when compared with sputum culture was statistically significant ($\chi^2 = 226.811$; P value < 0.0001).

Measures of diagnostic accuracy are summarised in table 4. Sensitivity of direct sputum smear microscopy was 88.8%; its specificity was 78.9%; PPV was 67.2%, NPV 93.6%, LR+ 4.22, LR- 0.14, DOR 29.9 and accuracy, 82%. The receiver operating characteristic curve for DSM in comparison with sputum culture as the gold standard is shown in figure 2.

Measure	Formula	Calculation	Estimate	95% CI
Sensitivity	TP/(TP + FN)	160/180	0.888	0.833 - 0.931
Specificity	TN/(FP + TN)	292/370	0.789	0.744 - 0.823
PPV	TP/(TP + FP)	160/238	0.672	0.608 - 0.732
NPV	TN/(FN + TN)	292/312	0.936	0.903 - 0.960
LR+	Sensitivity/(1 - specificity)	0.888/(1 - 0.789)	4.217	3.455 - 5.188
LR−	(1 - sensitivity)/specificity	(1 - 0.889)/0.789	0.141	0.092 - 0.210
DOR	LR+/LR−	4.213/0.141	29.94	17.283 - 53.298
Accuracy	(TP + TN)/(TP + FP + FN + TN)	160 + 292/550	0.82	0.787 - 0.853

Table 4: *Diagnostic accuracy measures of direct sputum smear microscopy compared to sputum culture as gold standard on 550 sputum samples examined.*

TP: True Positives; FP: False Positives; FN: False Negatives: TN: True Negatives; PPV: Positive Predictive Value; NPV: Negative Predictive Value; LR+: Positive Likelihood Ratio; LR−: Negative Likelihood Ratio; DOR: Diagnostic Odds Ratio; CI: Confidence Interval.

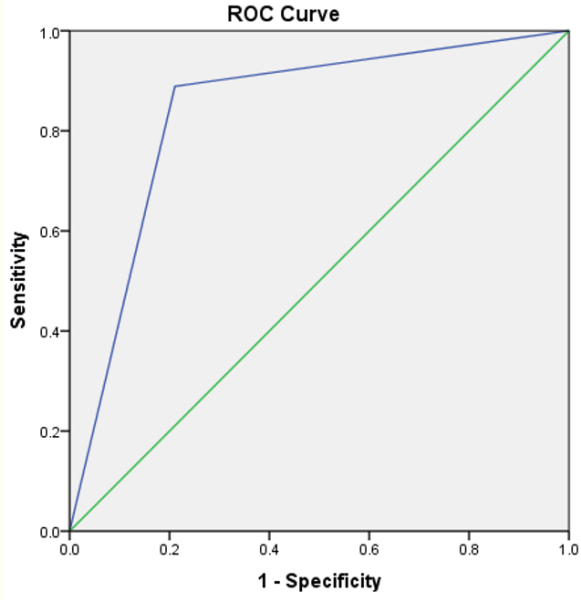

Figure 2: *Receiver operating characteristic (ROC) curve of direct smear microscopy based on culture as the gold standard. Area under curve = 0.893 (95% CI, 0.806 - 0.869), p < 0.001.*

Discussion

Tuberculosis is a major public health problem in the study population evidenced by the pulmonary TB case detection rate of 32.7% obtained by sputum culture. This rate is similar to 34.0% previously reported in Abia state, South-eastern Nigeria [8] but much less than 40.3% in Lagos, South-western Nigeria [19]. There was a preponderance of males in the study sample who had TB (60.6%) to their female counterparts (39.4%) which is consistent with the global male-to-female case notification ratio of 1.7 and results of local research [20,21]. Most countries share a similar sex pattern, having higher case notification rates for males than females even where there is equal health care access for both sexes [22]. The differences might be a reflection of difference in TB transmission dynamics in different parts of the world or TB being under diagnosed or under reported in females. Physiologically, it has been suggested that the male hormone, testosterone increases susceptibility to infection with mycobacteria [23]. However social roles and risk behaviours of males and females appear to have a greater effect. Men have higher levels of smoking and alcohol abuse particularly in high burden, low income countries [23,24] which lowers their immunity making them more susceptible to TB infection with higher TB prevalence, despite a higher prevalence of HIV infection in women in sub-Saharan Africa [25]. There is also social stigma associated with TB particularly in women; married females with TB disease are at greater risk of divorce, stigma and discrimination from in-laws. Unmarried females are also socially affected because having TB disease may reduce their chances of marriage [26].

The prevalence was high among the economically productive age group of 21- 40 years (65%) in this study. Tuberculosis in this age group tends to exert a huge economic burden on the individuals and their households as a result of lost income, resulting in huge societal economic losses also [27,28].

We found a TB/HIV co-infection rate of 18.9% in our study. Similar studies in Nigeria, conducted in Abia (8), Kano (9) and Lagos (19) states reported much higher rates of 48.0%, 38.4% and 72.0% respectively. Lower rates than we obtained have also been reported [29,30]. Human immunodeficiency virus infection causes reactivation of latent or dormant TB infection; it also causes rapid progression of recently and newly acquired TB infection, thus increasing TB incidence and prevalence [31]. The lower prevalence of TB/HIV co-infection obtained in this present study and others could be attributed to non-availability of facilities for routine TB culture in these areas.

The proportion of patients detected as positive for *M. tuberculosis* by direct sputum smear microscopy in this study was 29.1% but with the aid of culture, the detection rate increased to 32.7% indicating that 3.6% of study population would have inadvertently been excluded or missed if DSM alone had been used in diagnosis. HIV-related TB is often smear-negative and as our study has demonstrated, TB diagnosis in about 29.4% of the HIV-positive TB patients in the study population and this would have been missed using DSM only without including culture as a back-up investigation. Missed diagnosis by DSM compared to that of culture may be attributed to the quality of sputum used as well as the proficiency of laboratory scientists in reading the sputum smears thus influencing the result of the DSM. A missed diagnosis of smear-positive TB patient prolongs morbidity and can ultimately lead to death in an individual. It also places a heavy financial burden on the patients, their families and country as a whole [32].

Our findings also showed that, although culture produced a higher prevalence of 32.7% than DSM, this value was a lower positivity than should have been obtain as a result of contamination of the culture medium; about 6.9% contaminated growth would have increased the prevalence rate further. Requirements for appropriate media, high quality specimen and proper technique are crucial to bacteriological confirmation of TB. During processing of samples if decontamination is not carefully handled, on one hand, excess concentration of sodium hydroxide (NaOH) may kill the bacteria causing low yield of *M. tuberculosis* while on the other hand, a low concentration of NAOH allows for sample contamination by other organisms [33].

Our study demonstrated moderate sensitivity and specificity of sputum DSM for tuberculosis. Ideally; a diagnostic test should be 100% sensitive and 100% specific. Sensitivity and specificity of DSM depend on the quality of the sputum specimen and quality of smear prepa-

ration, staining and examination [34]. There is a wide variation in sensitivity of DSM reported in literature with figures as low as 20% and as high as 80%[34]. In this study however, sensitivity of DSM was 88.8% (95% CL 83.3% - 93.1%). This is comparable to results obtained in Kenya [35] and Indonesia [36] where DSM sensitivity was found to be 88.1% and 83.1% respectively but higher than 61.8% obtained in Tanzania [16]. DSM was also more sensitive in the HIV-negative TB patients than in the HIV-positive TB patients in our study [19]. Sputum samples from HIV-positive patients are often smear negative for AFB because of the low rate of caseation necrosis that leads to lower numbers of AFB in their airways [1]. This may result in poor quality sputum or non-sputum production from TB/HIV co-infected patients. Physical and chemical sputum processing methods such sputum concentration by centrifugation or sedimentation and use of bleach can improve the sensitivity of DSM [37] but these techniques have not been as successful in HIV-infected patients[38]. Furthermore, these sputum processing methods are potentially bio-hazardous and may not be available in low-resource settings due to their increased cost and complexity [37].

The specificity of DSM in this study was 78.9% when compared to culture. Sputum culture is the more sensitive and specific technique by which to detect TB compared to DSM. It is also necessary for confirming drug susceptibility in multi-drug resistant tuberculosis. However, the slow growth of the organism, sputum culture requires 4-6 weeks to produce a positive result on solid media. This coupled with necessary training, infrastructure, infection control and quality assurance requirements for culture make the use DSM more frequent in low resource countries [36]. Unfortunately, in our study, DSM diagnosed non-tuberculous mycobacteria as *M. tuberculosis* in 1.8% of the samples lowering its specificity. The diagnostic accuracy of DSM ability to discriminate tuberculosis infected persons from non-tuberculosis infected individuals was further analysed using a ROC curve. The area under the ROC curve was 0.839 (95% CI, 0.806 - 0.869) indicating that DSM has a lesser ability to discriminate between patients with or without tuberculosis than culture. Nyamogoba., *et al*. [35] reported a similar result in their study.

Conclusion

The findings from this investigation strongly suggest that the use of only DSM for TB diagnosis especially in communities with high HIV prevalence would produce unreliable and misleading results. Many TB patients would not be identified leading to missed diagnoses, delays in diagnosis and low treatment coverage with on-going transmission of TB in the community. Although culture is more expensive than DSM, it is necessary for culture facilities to be established in TB-DOTS treatment centres to enhance DSM for reproducibility and better estimation of TB burden in the country irrespective of the slow growth of *M. tuberculosis.* Adequate case finding and culture facility is very crucial as a backup for DSM to improve diagnosis, treatment and control of TB.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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