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RESPIRATORY INFECTIONS

Nanogold Assay Improves Accuracy of Conventional TB Diagnostics

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Abstract

Purpose TB nanodiagnostics have witnessed considerable development. However, most of the published reports did not proceed beyond proof-of-concept. Our objectives are to evaluate the diagnostic accuracy of a novel nanogold assay in detecting patients with active pulmonary TB based on results of BACTEC MGIT (reference test), and to compare its clinical performance to combined use of sputum smear microscopy (SSM) with chest X-ray (CXR).

Methods This is a case–control study that involved 20 active TB patients; 20 non-TB chest patients with a previous history of TB infection; 20 non-TB chest patients without a previous history of TB infection.

Results Sensitivity and specificity of TB nanogold assay were 95% and 100%, respectively, with diagnostic odds ratio (DOR) of 1053.0. ROC curve analysis yielded an area under curve (AUC) of 0.975. TB nanogold assay generated higher performance than combined use of SSM with CXR. The DOR and AUC differences were 996.0 and 0.125, respectively.

Conclusions Our study shows that TB nanogold assay is accurate, rapid, and holds the potential for use as an add-on initial test to improve accuracy of SSM and CXR in detecting patients of active pulmonary TB in developing countries. Future studies should involve larger sample size for further assessment of test accuracy.

Keywords Tuberculosis · Diagnosis · Gold · Nanoassay · Nanodiagnostics

Hesham El-Samadony and Hassan M. E. Azzazy have contributed equally to this work.

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Abbreviations

AUC	Area under curve
AuNPs	Gold nanoparticles
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CXR	Chest X-ray
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
EGP	Egyptian pound
NP	Nanoparticle
PA	Posterior–anterior
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic
SSM	Sputum smear microscopy
TB	Tuberculosis
US$	United States dollar
WHO	World Health Organization

Introduction

TB is ranked as the first cause of death from a single infectious agent for the last 5 years [1]. End TB Strategy targets ending the epidemic of TB by 2030. A fundamental milestone to achieve this goal is to optimize TB diagnosis by providing tools that are rapid, accurate, and affordable [1]. High tech-based assays have been recently introduced to TB diagnostic landscape—of which XpertMTB/RIF® system is the most powerful breakthrough [2], while others are still in the pipeline [3]. However, high cost and required sophisticated laboratory infrastructure hinder the implication of such excellent technologies in all developing countries of high TB burden. The current progress in TB diagnostic landscape does not outweigh the existing large gap in TB diagnosis [1] and conventional methods remain crucial [4].

Unique properties of nanoparticles have been employed in the detection of TB biomarkers [5]. To date, TB nanodiagnostics platform encompasses around 40 nanoassays that detect TB via DNA, antigen/antibody reaction, or metabolite-based approaches. Gold nanoparticles (AuNPs) have been the most-employed nanoparticles because of their exceptional properties [6]. Altogether, preliminary studies on TB nanodiagnostics reported accuracy of 80–100% and turnaround time of 2 min to 6 h. Despite the high potential, translation into clinical use has not been reached yet [7]. Recently, we developed a novel AuNPs-based colorimetric assay for detecting TB DNA in sputum in 2 h turnaround time, and with a detection limit of 11.2 ng TB DNA (see Online Supplement) [8]. In this study, we have evaluated sensitivity and specificity of TB nanogold assay in detecting patients with active pulmonary TB based on results of BACTEC MGIT (reference test). In addition, clinical performance of TB nanogold assay was compared to combined use of sputum smear microscopy (SSM) with chest X-ray (CXR). Finally, considerations to implement TB nanogold assay were assessed.

Materials and Methods

Study Design

This study follows two-gate case-control design with alternative diagnosis controls. The study is retrospective since BACTEC MGIT (reference test) was done before TB nanogold assay (index test). In addition, data collection and analysis were conducted after receiving results of the BACTEC MGIT. However, results of the TB nanogold assay were observed by an independent reader who was blinded to participants' data and results of BACTEC MGIT.

Participants

Group Enrolment

A total of 60 participants were recruited in consecutive series from ward department of Abbassia Chest Hospital, Ministry of Health, Cairo, Egypt, between 1 February 2016 and 30 June 2016. According to participants' diagnosis, participants were enrolled into two main groups (I) Case group: included 20 patients of active pulmonary TB; (II) Control group: included 40 patients who suffered from any chest diseases other than pulmonary TB (e.g., pneumonia, COPD). The control group was subsequently divided into two subgroups based on participants' medical history: IIa: included 20 TB-negative patients who had never been infected by pulmonary TB; IIb: included 20 TB-negative patients who had a previous history of TB infection but currently cured after receiving therapy. Old TB patients may persistently harbor TB DNA (of dead TB bacilli) in their sputum. This may undermine specificity of TB nanogold assay (DNA-based) and may lead to false-positive results [9]. This justified subgroup classification of controls.

Group enrollment (and subsequent sample collection) was initially based on medical history, clinical examination, results of sputum smear microscopy (SSM), and chest X-ray (CXR) findings. However, the final classification of participants was done when BACTEC MGIT results were recieved (reference test). The initial classification was needed owing to the long turnaround time of BACTEC MGIT system (14–42 days) [10], through which, TB patients (cases), whose diagnosis was based on positive SSM, must receive anti-TB therapy according to WHO and National TB Control guidelines [11, 12]. Such patients probably had negative smear conversion in response to therapy, and subsequently gave false negative result by nanogold assay if samples were collected after long lag time needed to report culture results. Hence, underestimating the sensitivity of nanogold assay.

Participants were initially classified as cases when tested positive by SSM for at least two sputum samples; controls when tested negative by SSM for three sputum samples. Group classification was considered inconclusive for participants who obtained positive SSM for only one sputum sample [11, 12].

Eligibility

Patients eligible for inclusion were consecutive adults (≥ 18 years) of both genders who could produce sputum. For cases, patients presented by symptoms suggesting active pulmonary TB (e.g., chronic cough, weight loss, appetite loss, night sweating, night fever and/or hemoptysis), with/without abnormal CXR findings, positive SSM, or past history TB infection. Yet, they tested positive with BACTEC MGIT. For controls, patients presented by any chest disease

other than pulmonary TB, with/without a past history of TB infection, and had negative BACTEC MGIT result.

We excluded: (i) patients who refused to participate in the study; (ii) active TB patients who, at time of recruitment, had already received anti-TB therapy for more than 7 days (since 22% of active TB patients had negative sputum conversion after 2 weeks of therapy [13], hence, probably underestimating sensitivity of TB nanogold assay through obtaining false negative results); (iii) patients who could not produce sputum or submitted inappropriate sputum samples, e.g., bloody, watery (saliva), scanty (volume < 5 ml); (iv) extra-pulmonary TB patients.

Test Methods

All participants were subjected to the following: (i) full history taking (reported from admission sheet); (ii) clinical examination; (iii) chest X-ray (CXR) (PA view); (iv) sputum smear microscopy (SSM) examination for three successive sputum samples using Ziehl–Neelsen stain processed according to WHO protocol [14]; (v) submitting one early morning sputum sample collected in a sterile cup and utilized as follows: 1.5 ml was examined by TB nanogold assay, and the rest of the sample (≥ 3.5 ml) was used for TB sputum culture using BACTEC MGIT.

TB Nanogold Assay

See Online Supplement for fabrication concepts and detailed procedure.

First, DNA was extracted from sputum sample and then stored at − 20 °C for further assay steps when culture results were obtained. Second, PCR was done—after receiving culture results—targeting IS6110 TB DNA. Finally, the red solution of AuNPs was added to test ingredients including PCR product of sample, salt, and Tris–HCl. The colorimetric result of TB nanogold assay was observed by naked eye after 2 min of adding gold. Positive result was defined as preserved red color of test solution; negative result was defined as red-to-violet color shift of test solution (See Online Supplement, Figs. 1, 2).

Reference Test

BACTEC MGIT TB System was adopted as the reference test of the study. Samples were processed for culture within 2 h of collection. Test procedure and result reporting were conducted according to the manufacturer's protocol [10].

Statistics

Sensitivity and specificity of TB nanogold assay, and combined use of SSM with CXR were analyzed against results of BACTEC MGIT using ROC curve analysis and diagnostic odds ratio (DOR). For statistical analysis, inconclusive results of SSM and CXR (i.e., positive SSM for only one out of three sputum samples) were grouped with positive results considering high clinical cost of missing a TB diagnosis [15]. All statistical testing was performed using MedCalc Statistical Software® 2017. No missing data was encountered for TB nanogold assay, SSM, CXR, or BACTEC MGIT.

The minimum sample size required for this study was 60 participants and the maximum was 159 participants considering that: type I error was 0.05; the study power was 80%; the

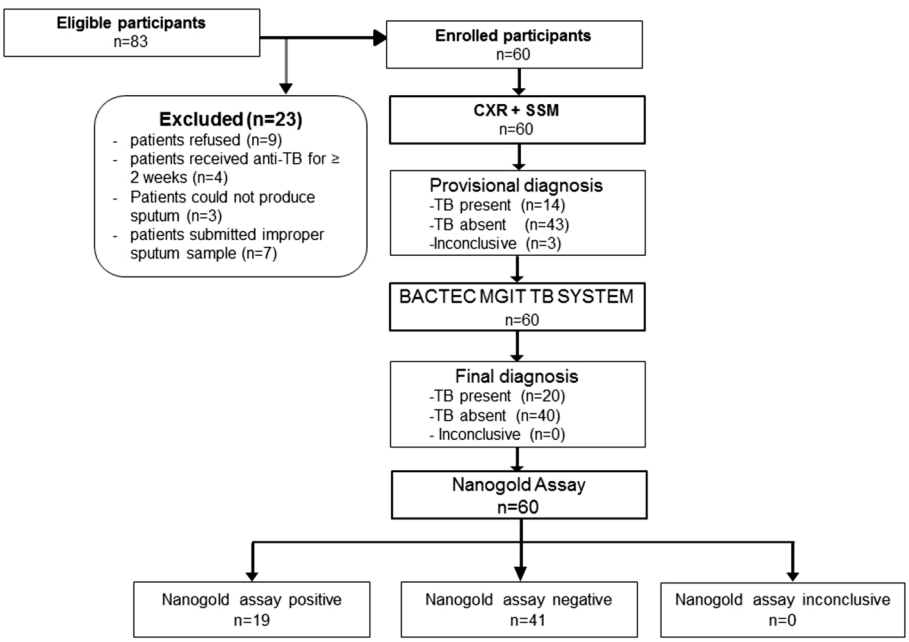

Fig. 1 Diagram of participants flow through the study with diagnosis

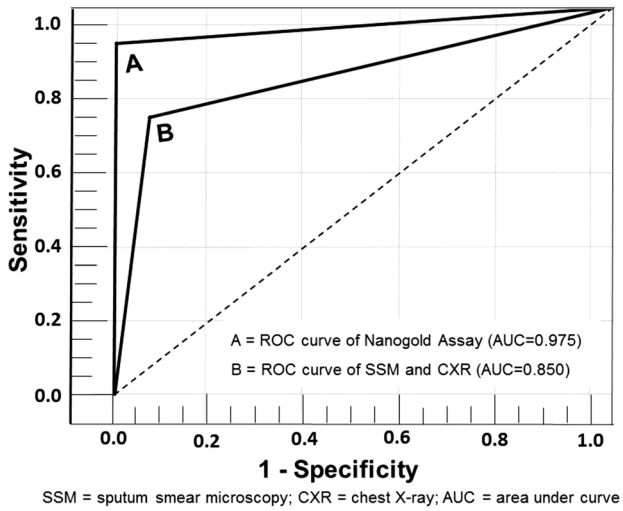

Fig. 2 Receiver operating characteristics (ROC) curve for diagnostic accuracy of nanogold assay, and sputum smear microscopy with chest X-ray. ROC curve analysis of diagnostic accuracy generated an AUC of 0.975 for TB nanogold assay, and 0.850 for SSM with CXR. The AUC difference was 0.125

expected area under the ROC curve would be 0.73; the ratio of the sample size between case and control groups was 1/2. Accordingly, sample size for this study was 60 participants.

Results

Participants

Between 1 February 2016 and 30 June 2016, 83 eligible participants were invited to join this study. 60 participants completed the study with a completion rate of 72%. Figure 1 shows a flow diagram that illustrates stages of the study and diagnosis outcomes. Table 1 shows demographic and clinical characteristics of study participants. For case group, the sputum sample utilized in TB nanogold assay and BACTEC MGIT was collected before starting anti-TB therapy in 7/20 participants (35%), while 13 patients obtained sputum samples after starting anti-TB therapy with time interval of 2.5 ± 0.7 days (range 1–5 days).

Test Results

SSM and CXR

Table 1 shows results of SSM and CXR for all study participants, while Table 2 shows diagnosis of all study participants based on BACTEC MGIT results, along with clinical characteristics and radiological findings. SSM with CXR obtained sensitivity of 75% (95% CI 50.90–91.34%) and specificity of 95% (95% CI 83.08–99.39%) (Table 3). The diagnostic odds ratio was 57.0 (P < 0.0001). ROC curve analysis obtained AUC of 0.850 (95% CI 0.734–0.929) (Fig. 2).

Table 1 Demographic data, clinical characteristics, chest X-ray findings, and results of sputum smear microscopy for all study participants

Patient characteristics	n (%)
Mean age, years (range)	50.3 ± 10.97 (30–68)
Men	45 (75.0)
Clinical presentation	
Cough and expectoration	60 (100.0)
Toxic symptoms	37 (61.7)
Hemoptysis	31 (551.7)
Dyspnea	33 (55.0)
Chest wheezes	16 (26.7)
Chest pain	15 (25.0)
Past history of TB/duration since TB infection	26 (43.3)/(14.5 ± 11.3 years) (range 1 month to 40 years)
Physical examination	
Clubbing	12 (20.0)
Cyanosis	8 (13.3)
Tracheal shift	3 (5.0)
Chest auscultation	
Diminished air entry (localized)	12 (20.0)
Wheezes	25 (46.7)
Crackles	16 (26.7)
Chest X-ray findings	
Normal	5 (8.3)
Abnormal	55 (91.7)
Consolidation	14 (31.7)
Reticulations	21 (35.1)
Lung hyperinflation	13 (21.7)
Nodule(s)	7 (11.7)
Cavity(s)	6 (10.0)
Ring shadows (bronchiectasis)	5 (8.3)
Effusion opacity	4 (6.7)
Destructed lung	2 (3.3)
Hilar shadow (adenopathy)	4 (6.7)
Sputum smear microscopy	
Negative	46 (71.7)
Positive	17 (28.3)
3/3 samples	6 (15.0)
2/3 samples	8 (8.3)
1/3 sample	3 (5.0)

TB Nanogold Assay

TB nanogoldassay obtained 95% sensitivity (95% CI 75.13–99.87%), and 100% specificity (95% CI 91.19–100.00%) (Table 3). The diagnostic odds ratio of TB nanogold assay was 1053.0 (P < 0.0001). ROC curve analysis obtained AUC

Table 2 Diagnosis of study patients based on clinical data, and BACTEC MGIT results

Diagnosis	n (% group)
Cases (n = 20)	
Fresh case	12 (60.0)
TB relapse	6 (30.0)
TB defaulter	2 (10.0)
Controls (n = 40)	
COPD	9 (22.5)
Pneumonia	6 (15.0)
Asthmatic bronchitis	5 (12.5)
Infected bronchiectasis	4 (10.0)
ILD-acute exacerbation	2 (10.0)
Post TB lung fibrosis/extensive destruction	4 (5.0)
Lung abscess	3 (7.5)
Lobar/segmental collapse	3 (7.5)
Pleural effusion	4 (10.0)

Table 3 Diagnostic accuracy of sputum smear microscopy (SSM) with chest X-ray (CXR), versus TB nanogold assay

Studied Test(s)	BACTEC MGIT (reference test)		Total
	TB positive	TB negative	
SSM + CXR			
Positive	12	2	14
Negative	5	38	43
Inconclusive	3	0	3
Total	20	40	60
TB nanogold assay			
Positive	19	0	19
Negative	1	40	41
Total	20	40	60

of 0.975 (95%CI 0.897–0.998) (Fig. 2). TB nanogoldassay generated higher clinical performance than SSM and CXR with absolute difference in sensitivity and specificity of 20 and 5 percentage points, respectively, DOR difference of 996.0 ($P < 0.0001$), and AUC difference of 0.125 (95% CI 0.0288–0.221) (Fig. 2).

Discussion

TB diagnostic landscape still lacks the ideal TB testing given the inherent shortcomings of conventional diagnostics along with high cost and technical complexity of new high tech-based tests [16]. Nanoparticles possess unique properties that allowed their utilization in detection of TB biomarkers [7]. Recently, we have developed a sputum-based tool for TB diagnosis using bare AuNPs. Our proposed assay succeeded to detect TB DNA in 2 h turnaround time, and with a detection limit of 11.2 ng/μL TB DNA (see Online Supplement). Here, we have evaluated diagnostic accuracy of TB nanogold assay in detecting patients with active pulmonary TB using BACTEC MGIT culture as the reference test. In addition, the accuracy of TB nanogoldassay has been compared to that of SSM combined with CXR.

Our data demonstrated that TB nanogold assay outperforms BACTEC MGIT and SSM with CXR, yet, in a shorter turnaround time (2 h). TB nanogold assay achieved 95% sensitivity, 100% specificity, 1053.0 diagnostic odds ratio (DOR), and 0.975 ROC AUC. In addition, TB nanogold assay performed better than combined use of SSM with CXR. The DOR and AUC differences were 996.0 and 0.125, respectively.

TB nanogold assay succeeded to detect 19/20 active TB cases including 4 SSM−/culture+ and 15 SSM+/culture+ TB patients. In addition, it confirmed TB diagnosis of three cases whose diagnosis was inconclusive based on SSM (one positive sample out of three) and CXR (non-specific findings). In terms of specificity, TB nanogold assay excluded TB infection in 40/40 non-TB chest patients including five patients who gave false-positive SSM, and 20 patients who had a past history of active TB disease.

In terms of cost-effectiveness, TB nanogold assay is PCR based which increases cost and complexity of testing. On the other hand, AuNPs are synthesized using a simple protocol that does not require sophisticated devices or special instruments [17]. Despite using a precious metal, only minute quantities of gold are required to synthesize hundreds of milliliters of gold solution, of which, only 30 microliters are needed per test. Based on our experience, synthesized gold solution preserves its properties and is valid for use for up to 1 year when properly stored [18]. In addition, using bare AuNPs without any modification minimizes fabrication cost in contrast to many reported TB nanodiagnostics for which nanoparticles were modified with bio-targeters (e.g., oligonucleotides, antigen, antibodies, etc.) [7]. For assay implementation, TB nanogold assay requires a single-patient visit compared to SSM which necessitates submitting three sputum samples on two consecutive days. Given its straightforward procedure and simple colorimetric strategy of detection, implementing TB nanogold assay only requires minimal training of laboratory personnel. Moreover, TB nanogold assay does not require special building space, laboratory infrastructure, equipment, nor regular maintenance—compared to the device of GeneXpert TB test which is sensitive to heat, humidity, and dust, and requires a stable electrical supply and regular calibration [19, 20]. Being a conventional PCR-based test, TB DNA amplification could be done

using low-cost, simple-to-operate, portable (open source) thermal cyclers with laboratory capacity of microscopy center level [21].

The cost of kits and reagents for TB nanogold assay is approximately US$10/test which is almost equal to the discounted price of GeneXpert cartridge (US$9.98/test) offered to 145 developing countries of high TB burden, based on an agreement between *Cepheid* and several charities [22]. Yet, implementing TB nanogold assay does not require significant cost and installation infrastructure required for Xpert TB system [23]. Although the WHO recommends using Xpert TB test as an initial testing for all TB suspect patients [1, 20], cost consideration hinders adopting this recommendation in low- and middle-income countries where TB burden prevails [24]. Taking Egypt as an example, according to National TB Control Guidelines [12], using Xpert TB test is restricted to certain clinical indications (e.g., TB relapse, delayed sputum conversion after therapy, extrapulmonary TB) owing to limited number of available cartridges which are donated to National TB Program by The Global Fund [25]. In addition, the private medical sector in Egypt is not eligible for the concessional price of Xpert TB assay [21] and charged US$55.98 (1000 EGP) per test. Given these considerations, TB nanogold assay could operate as an add-on initial diagnostic tool for TB suspect patients in developing countries.

However, TB nanogold assay has limitations. AuNPs are extremely sensitive to concentration/pH of other test ingredients (NaCl and Tris–HCl). Accordingly, any pipetting error could alter assay results. In addition, the test is performed through an open system with frequent manipulation of PCR product, which increases risk of cross-contamination.

This study had two limitations. Regarding design, the study is open-label since data were collected and analyzed after receiving results of BACTE MGIT (reference test). This consequently could bias estimates of diagnostic accuracy. However, colorimetric result of TB nanogold assay was observed by an independent reader who was blinded to BACTEC MGIT results and participants data [26]. In addition, the study involved a small sample size which could inflate accuracy estimates.

Conclusions

The study shows that TB nanogold assay has the capacity to use as an add-on initial test to improve accuracy of SSM and CXR in TB diagnosis in developing countries—given its high accuracy, short time-to-result, and accessible requirements for implementation. This is the first time to assess clinical performance of TB nanogold assay in clinical settings. Future studies should involve larger sample size for proper assessment of test accuracy. Further research is required to adopt low-cost technologies for DNA amplification (e.g., isothermal amplification techniques [27], portable thermal cyclers [21]) to boost assay affordability. Assay should be optimized to develop a fully integrated closed system to reduce cross-contamination and provide maximal simplicity of the end user in resource-limited countries.

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Compliance with Ethical Standards

Conflict of interest HMEA is a co-founder of D-Kimia, LLC, a novel diagnostic solutions company and author of patents on use of gold nanoparticles for detection of infectious agents. Other authors declare no competing interest.

Ethical Approval The Research Ethics Committee of the Egyptian Ministry of Health approved study protocol (Approval No. 31-2014/8).

Informed Consent A written informed consent was obtained from all enrolled patients.

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