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DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* FROM PULMONARY SPUTUM SAMPLE USING SPION MEDIATED DNA EXTRACTION METHOD

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ABSTRACT: A *Mycobacterium* DNA extraction protocol utilizing SPION developed and compared with the conventional and commercial kit method. The quality, quantity, and feasibility of the recovered DNA from all four different methods towards various molecular techniques were observed. In our work, morphology, bonding and composite of SPION is determined by various characterization techniques such as XRD, FT-IR, VSM, UV-vis spectroscopy and FE-SEM. XRD analysis confirms the crystallite size and phase purity of the synthesized material. The average crystallite size obtained were ~ 9.7 nm for SPION, prepared by a Co-precipitation method. FT-IR spectrum also confirms the formation of SPIONs. UV-vis spectrophotometry and gel electrophoresis analysis resulted in highest MTB DNA yield (985.3 ng/µl) and the purity was measured by the ratio $A_{260} / A_{280} = 1.89$. The comparative study suggested that the protocol developed in this report is a unique cost effective and highly pure. Further, Real-time PCR also confirms the superiority of our technique.

KEYWORDS: MTB– *Mycobacterium tuberculosis*, DNA- Deoxyribonucleic acid, SPION- Super paramagnetic iron oxide nanoparticle, PCR-Polymer chain reaction.

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1. INTRODUCTION

In 2016, it is declared the world health organization (WHO, 2016), that tuberculosis (TB) problem is increased, it is threatening to the public health. 1.5 million people suffered and died because of TB. The toll comprised 890,000 male, 480,000 female and 140,000 kids [1]. Worldwide 9.6 million people were predictably fallen ill with TB in 2016:5.4 million males, 3.2 million females, and 1.0 million kids. Globally 4.6 million new cases of TB were reported to WHO fewer than two third (63%) of the 9.6 million human being expected unwell with the sickness. Which means globally 37% of latest cases have been un-diagnosed or were now not reported [1]. There may be an urgent requirement for fast and price effective diagnostic approach [2]. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in lots of different bacteria, being hydrophobic, waxy, and rich in mycolic acids [3]. Pulmonary tuberculosis represents around 85% of TB cases, at the same time as much extra pulmonary TB is lower in common (15%) [4]. There are a variety of methods were used such as clinical findings, X–ray chest, Microscopy-AFB, Tuberculin sensitivity test (PPD), microbiological culture method, PCR and Histopathology [5]. The standard TB diagnostic method in developing countries continues to be the sputum smear microscopy [6]. It was developed in 1880 and has remained almost the identical given that it relies on the presence of acid-fast bacilli (AFB) in the stained smear [7]. The first line diagnostic method for MTB is smear microscopy it relies on staining of the acid-fast bacilli using Ziehl Neelsen (ZN) stain. It is fast and cheap so used heavily in poor-resource countries for TB diagnosis and monitoring the patients receiving anti-tuberculosis antibiotics. However, this technique required >10,000 bacteria/ml present in the sample, to allow bacilli detection by smear microscopy. Misdiagnosed patients by smear microscopy due to their low bacterial load transmit the bacilli to their surrounding community also; it does not give information about the bacterial type [7, 8]. Most of the physicians still based their diagnosis in TB cases on direct sputum smear microscopy for ZN staining and culture for *Mycobacterium tuberculosis* (MTB) [9]. Microbiological culture when compared with liquid and solid culture as the gold standard method, but it requires >35 days. The conventional chloroform-phenol method of DNA extraction is a poisonous, time consuming, multi-step procedure and the residual phenol can be inhibited to PCR amplification [10]. A TB detection method remains a challenging for both clinicians and microbiologists because of the low sensitivity of conventional acid-fast staining method. To reduce this delay in diagnosis of TB, molecular base method for TB diagnosis is highlighted under the new branch in medical microbiology is a polymer chain reaction (PCR). It is accurate, rapid, safe and reliable method for reproducible identification of MTB infection. The disadvantage of PCR is its high cost, molecular lab infrastructure and it require skilled medical technologist [10]. The success of final amplification and detection of Nucleic Acid Amplification test (NAAT) which depends on successful DNA extraction from pulmonary sputum samples [11]. SPION (superparamagnetic iron oxide nanoparticle) primarily based MTB DNA extraction technique is

straightforward to synthesize, it provides a huge surface region, do not interfere the structure and properties of DNA. Magnetic properties and biocompatibility, is helpful in magnetic extraction of DNA [12]. Magnetic adsorption techniques used for isolation of DNA from pulmonary sputum samples. We report a SPION mediated MTB DNA extraction from positive sputum samples. It proves to be a rapid, cheap, and done in a single test tube, without any toxic chemicals and enzyme substances. Extracted MTB DNA samples from this technique are worthy for Real-Time PCR detection. [13,14]. SPIONs were utilized in various biomedical applications for increasing sensitivity of DNA-based biosensors, magnetic resonance imaging (MRI) contrast agent, target drug delivery and imaging, cellular tracking, magnetic separation technologies, imaging probe, microfluidic device etc. [15]. To conquer these limitations, we have developed advanced SPION-mediated DNA extraction approach; therefore, it proves to be a rapid less expensive and achieved in a single test tube. Extracted DNA from this method are pure and worthy for real time PCR detection.

2. MATERIALS AND METHODS

2.1 Materials

Analytical grade chemicals were used for preparation of SPION for isolation of DNA mainly Ferrous chloride ($FeCl_2$) and Ferric chloride ($FeCl_3$) purchased from Molychem India, Sodium chloride (NaCl), Tris- EDTA, sodium dodecyl sulfate (SDS), Sodium hydroxide (NaOH), NALC (N-acetyl-L-cysteine) and Polyethylene glycol (PEG, MW 8000), were purchased from Thomas Baker.

2.2 Synthesis and Functionalization of SPION

The raw material SPIONs were prepared by conventional co-precipitation method as per previously reported method [15]. The Co-precipitation technique was used to synthesis SPIONs. Ferrous chloride ($FeCl_2$) and Ferric chloride ($FeCl_3$) were used as precursors for the reaction in 2:1 (v/v) proportions. In this process, the salt solution of the required metallic elements is reduced by 0.1 NaOH solutions. The reactants when mixed at a temperature of 90˚C. The appearance of black precipitate indicated the formation of iron oxide nanoparticles [16]. The precipitate was separated by using external magnets and washed thoroughly with double distilled water. The possible reaction taking place showed below:

$$2Fe^{3+} + Fe^{2+} + 8OH \rightarrow Fe_3O_4 + 4H_2O \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

2.3 Functionalization of SPION

Cetyltrimethyl ammonium bromide (CTAB) coated SPIONs were taken into a round bottom flask and aqueous ammonia, ethanol, and double distilled water was added into the flask. The mixture is allowed to stir and was added to its TEOS. Nanoparticles of Fe_3O_4 were collected through magnetic separation. Finally, they were isolated by centrifugation, washing several times with double distilled water and drying [17, 18].

2.4 Characterization of SPION

SPIONs were studied for their structural, morphological and magnetic properties using X-ray

Diffractometry (XRD), FTIR (Fourier Transform Infrared spectroscopy), VSM (vibrating sample magnetometer), and FE-SEM (Field emission scanning electron microscope). Phase identification and structural analysis of SPION were studied by XRD (Rigaku D/MAXIIA diffractometry) with Cu Kα radiation (λ=1.5406Å) operating at 40 KV and 40 mA in the 2θ range from 20° to 80°. The crystallite size of the synthesized product was estimated from the full-width at half-maximum 9 FW of the strongest diffraction peak by using the Scherrer's formula.

$$D = \frac{0.9\ \lambda}{\beta \cos\theta} \qquad \text{----------} \qquad (2)$$

Where D is the crystalline size, λ is the wavelength of Cu-Kα radiation, β is the FWHM and θ is the Diffraction angle of the strongest characteristic peak. The SPION were used to get FTIR spectra were collected on Alpha ATR Broker (Eco Model) at range 400-4000 cm^{-1} at room temperature for analysis to verify the Fe_3O_4 [18]. A VSM (vibrating sample magnetometer) was employed to measure the magnetic behavior of SPION the VSM curve clearly indicates the superparamagnetic nature of SPION.

2.5 Collection and processing of pulmonary sputum sample

Sputum samples were collected from D. Y. Patil hospital Kolhapur, in a sterile container. Samples are pre-treated with NALC (0.5 % N-acetyl-L-cysteine) and 2% sodium hydroxide for digestion and decontamination. In our protocol, we have used NALC method, which is approved by WHO. This method also helps to free the bacilli from the mucus cells in which they may be embedded in sputum. Total twelve pulmonary sputum samples are treated with NALC method. [19]. Microscopic acid-fast staining direct and concentrated smears are prepared from sputum samples. In brief, Samples are mixed with NALC-NaOH solution in a vortex mixture, centrifuged at 3000 rcf for 20 min at 4°C. The supernatant decanted, and transferred the sediment in 2 ml of phosphate buffer pH 6.8. The sediment used for AFB staining and DNA extraction [20].

2.6 Microscopic observations using acid-fast staining and Auramine O/Rhodamine stain.

For ZN (Ziehl-Neelsen method) staining Carbol fuchsin was covered on the slide and heated until steam arose, and it was allowed to stain for 5-10 minutes [21]. The slide was decolorized with 3% v/v acid alcohol or 20% sulfuric acid for 1 minutes. The slide was counterstained with methylene blue (0.5% w/v) for 2 minutes and washed with tap water. Air dry and observed under oil immersion objective for acid fast bacilli [22]. For auromine Auramine O / Auramine-Rhodamine stain**.** In this method primarily flourochrome stain auramine O was applied for 15 minutes and secondary stain 0.5% potassium permanganate for 2 minutes. Using LED-FM microscopy acid fast bacilli are seen [23].

2.7 Extraction and purification of MTB

a) Chloroform-phenol method: DNA was isolated from sputum sample using a standard method described elsewhere. DNA was finally suspended in 50 µl TE buffer and stored at -20°C [24, 25].

b) Commercial kit method: DNA isolated from sputum sample using the spin column method according to the manufacture's instructions. DNA was finally suspended in 50 µl TE buffer and stored at -20°C [25].

c) Extraction by using SPIONs: 200 µl of concentrated liquefied sputum were centrifuged for 15 minutes at 13000 rcf at (4ºC). Pallet suspended in 200 µl nuclease free water, vortex and boiled at 100ºC for 20 minutes, then cool to room temperature. Supernatant transferred into new 1.5 ml sterile micro centrifuge tube, 200µl lysis buffer (10mM Tris-HCl, 0.5 mM EDTA, 10%SDS, pH7.6) and 10µl proteinase-K (20 mg/ml) were added and incubated in a water bath at 56 ºC for 2hrs. Sample centrifuged at 10,000 rcf at 4ºC for 20 minutes. Supernatant was transferred to a new test tube containing 500 µl binding buffer (25% polyethylene glycol 8000 MW) and 100 µl SPIONs. Mixed by gently inverting and allowed to stand at room temperature for 5 minutes. Using external magnet supernatant was removed. The magnetic pallet washed with buffer (cold 70% Ethanol) and repeated for 2 to 3 times. Add 100 µl elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) to elute bound DNA by incubation at 56 ºC for 5 minutes with gentle agitation. Centrifuge at 13000 rcf for 5 min and collect 50µl elute in the new micro-centrifuge tube. A buffer containing the extracted DNA was transferred carefully into a fresh micro centrifuge tube and stored at -20°C.

d) Commercial magnetic bead extraction method: DNA was isolated from sputum sample using the magnetic beads according to the manufacture's instructions; nucleic acids are re-suspend in the elution buffer. DNA was transferred carefully into a fresh micro centrifuge test tube and stored at -20 °C [26].

2.8 Assessing the purity of DNA by using UV-vi's spectroscopy.

The purity and yield of the extracted DNA were analyzed using UV-vi's spectroscopic assessment, where A_{260}/A_{280} ratio gives protein contamination. The concentration of DNA can be determined by the equations 1 OD_{260} unit = 50 µg/ml for double stranded DNA [26, 27].

2.9.1 Real time PCR

Real time PCR was carried out by using extracted MTB DNA samples to check the compatibility towards the molecular technique. All extracted DNAs were submitted for SYBR green based real time PCR. The probes and primers were purchased from Genome Diagnostics Pvt. Ltd. (Accession number LC005454.1.) The reaction system contained 25 µl of total sample volume, in which 12.5 µl of MTB complex super mix, 2.5 µl Mg.Sol., MTB complex, internal control 0.5µl, Add 10 µl DNA to appropriate tube. Finally, insert the tubes in the thermal cycler Rotor Gene 2000/3000/6000, (Corbett Research Australia). [27] The reaction condition was 10 min.at 95°C: 45 cycles of 20 s at 60°C and 15 s at 72°C.

2.9.2 Agarose Gel Electrophoresis

Agarose gel (2%) electrophoresis was carried out using horizontal gel electrophoresis unit. All the

extracted DNAs was analyzed and observed under the gel image system (Syngene USA). The running buffer was 1 X TAE; electrophoresis was carried out at 60 mA at 100-120V for 2 hrs. DNA [28].

3. RESULTS AND DISCUSSION

3.1 Structural analysis of SPION

The XRD patterns of SPION were studied by X-ray diffractometer and the patterns were shown in Fig.1. The SPIONs displays diffraction peaks at (2θ= 30.23˚, 35.73˚, 43.15˚, 53.69˚, 57.46˚, 63.0˚) assigned to (220), (311), (400), (422), (511) and (531). The peaks are consistent with the database in standard JCPDS file No. 19-629 and reveal that resultant nanoparticles were pure SPION. The crystallite size of SPION was estimated from FWHM of the most intense peak (311) using Debye-Scherrer equation. The average crystallite size was found to be ~ 9.7 nm for SPIONs prepared by a Co-precipitation method using NaOH as a reducing reagent.

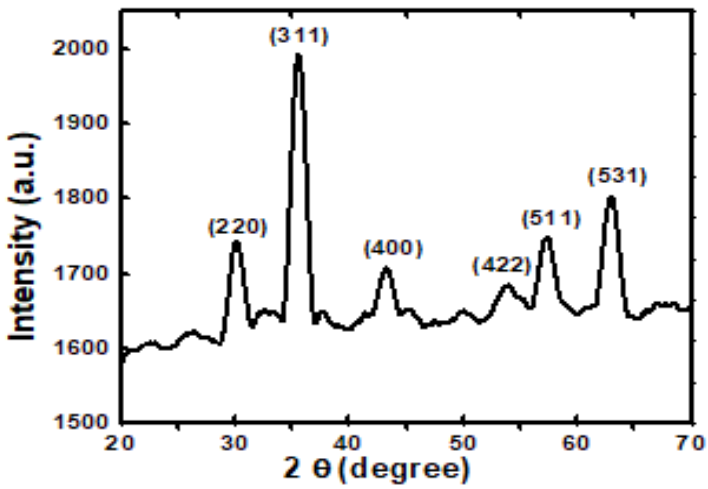

Fig 1. X-ray diffraction of SPION

3.2 FT-IR analysis of SPION

SPION FT-IR spectra in the range of 400 to 4000 cm^{-1} Fig.2 shows for the magnetite's, the band at 567 cm^{-1} corresponds to the vibration of the Fe- O bonds vibration related to the magnetite phase. Additional, the peaks at 1626 cm^{-1} and 3407cm^{-1} can be attributed to the stretching vibration of the hydroxyl groups on the surface of the SPION. The bands at 1461 cm^{-1} and 1626 cm^{-1} are assigned to the asymmetric and symmetric stretching vibrations of the carboxyl group (Coo^-) separately. The band at 2852 cm^{-1} and 2921 cm^{-1} correspond to the asymmetric and symmetric CH_2 stretching vibration of O-H. These results are in confirmation with the previous reports of SPIONs [29].

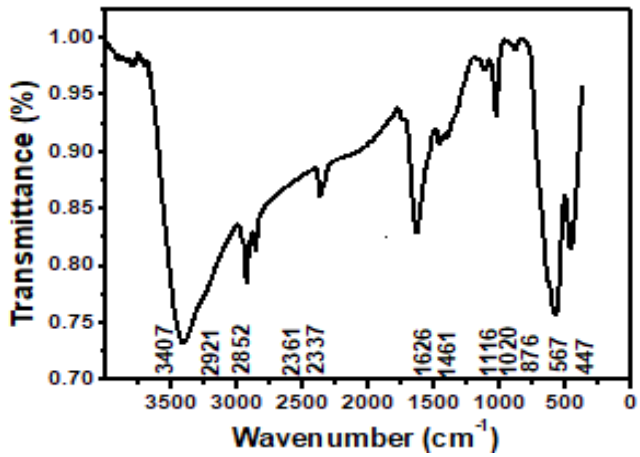

Fig 2. FT-IR of SPION

3.3 Magnetic measurements of SPION

The magnetic properties of SPION were studied using a vibrating sample magnetometer. The room temperature M–H curve of samples is shown in Fig 3. It can be seen that the M_S Value of the sample is 84 emu g^{-1}. The vibrating sample magnotometer (VSM) curve clearly indicates the superparamagnetic nature of the material which is highly desirable for biomedical operation because larger magnetic particles aggregated after exposure to a magnetic field [29].

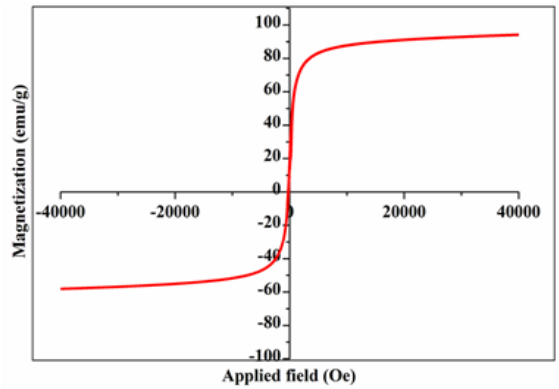

Fig 3. VSM of SPION

3.4 Morphological Analysis of SPION

Field emission scanning electron microscope (FE-SEM) images of SPION showed a high degree of agglomeration due to dipole-dipole interaction and spherical and granular in shape. SPIONs has strong magnetic dipole-dipole interaction, hence are attracted strongly and form big clusters, causing larger particle size shown in Fig 4.

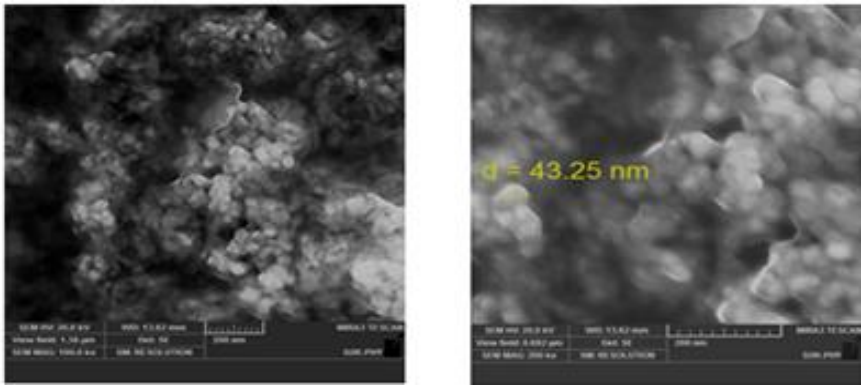

Fig 4. FE-SEM of SPION

4. Microscopic detection methods for MTB

4.1 ZN staining

Direct smear, examination of pulmonary sputum samples for AFB (Acid-fast bacilli) by ZN stained smear technique applied for the diagnosis of pulmonary TB. However, this technique required minimum >10,000 bacteria/ml present in the samples, to allow bacilli detection by smear microscopy [30]. All smears of the ZN stain observation revel AF-positive smear grade (+ + +) according to RNTCP guideline and the results are confirmed by microscopic observation which shown in Fig 5 (a).

4.2 The Flourochrome stain: The flourochrome dyes for *mycobacteria* are Auramine O/ Auramine Rhodamine apply to stain the smears for screening of AF bacilli in sputum [30]. The results are confirmed by LED-FM (Light Emitting Diode fluorescent microscopy) which was shown in Fig5 (b).

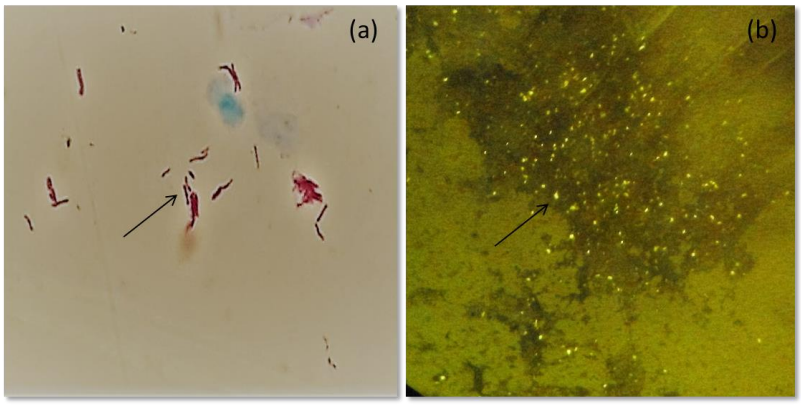

Fig 5. (a) ZN stain, acid fast bacilli red color. (b) Auramine O stain yellow color acid fast bacilli.

4.3 SPION assisted isolation of DNA

Magnetic adsorption techniques have been used for isolation of DNA from pulmonary sputum samples. Here, magnetic particles SPION were used for magnetic separation as well as solid phase support to adsorb DNA. It provides a higher surface area for adsorption of biomolecules and allows a greater response to the applied magnetic field and easy separation and manipulation of SPION. Magnetic particles binding with DNA to the surface of SPIONs are due to the electrostatic interaction between positively charged SPIONs and negative charged DNA molecule. Surface to volume ratio

was very high, and increase rapidly as the relevant dimension decreases for a 3 nm diameter, particles approximately 90% of the atoms reside on the surface. DNA was the molecule with a backbone of phosphodiester linkages hydroxy and amino groups present on the surface of SPIONs. It reacts with molecules bearing hydroxyl, carboxyl, phosphate, and sulfate groups through formation of hydrogen bonds. Phosphate groups were present in phosphodiester bond, and these were responsible for imparting a negative charge to DNA [31]. Electrostatic and hydrogen bonding were responsible for adsorption of DNA, the presence of protonated amino group $-NH_3^+$ and hydrogen bonding and carboxyl group with DNA.

4.4 Evaluation of extracting DNA and its comparison with SPION methods

There are numerous techniques for isolation of MTB DNA from sputum sample each method having its own profits as well as limitations. MTB DNA extraction is usually affected by various factors such as incomplete cell lysis, adsorption, desorption techniques to a particular material, chemical substances, and sample pre-treatment process. The quality, quantity and timing process of the extraction technique obtained by SPION method were compared with conventional chloroform-phenol and commercial kit base methods [32].

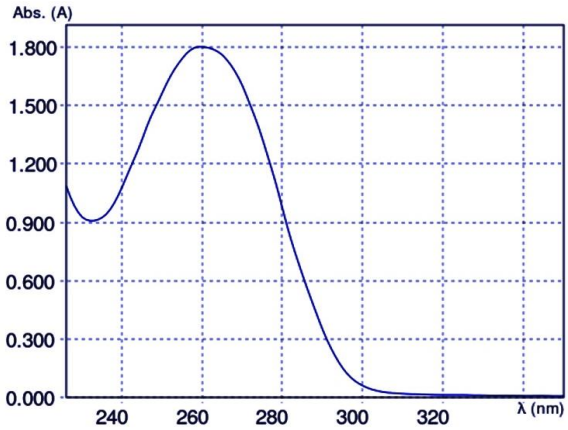

Fig 6. UV-visible spectra of eluted MTB DNA by SPION.

The purity of DNA sample was measured by A_{260} / A_{280} ratio, with absorption spectrum from 220-350 nm wavelength measured by using bio-spectrophotometer (Cary 60 Agilent Technology, USA). The concentration of the DNA in the sample was determined by the equations (One OD $_{260}$ Unit =50 µg/ml for double stranded DNA. The average A_{260} /A_{280} ratio and yield of SPION recovered DNA was approximately 1.8 to 2.0 indicates, least protein contamination, obtained by SPIONs [32]. By comparing the ratio between conventional and commercial kit methods table 1. Shows our novel DNA purification method using SPION are better than other method, the amount of eluted MTB DNA produced highest MTB DNA yield 985.3 ng/µl. The Absorbance A_{260}/A_{280} ratio is 1.72 and 1.87 which indicates DNA is pure and free from protein. [33]. Purity and yield of the

extracted DNAs were measured by UV-visible bio-spectrophotometer, which shows in (Fig 6).

Table 1: DNA isolation and quantification of results by Bio-spectrophotometer (USA)

Method	Sample	Yield of DNA (ng/µl)	A_{260}/1mm	A_{280}/1mm	A_{260}/A_{280}	Time requires	Cost/test/ USD	Reference
Chloroform-	1	65.9	0.131	0.053	2.21			[25]
Phenol	2	66.9	0.133	0.055	2.24	6hrs	0.5$	
	3	96.9	0.194	0.087	2.23			
Commercial	1	315.5	0.631	0.409	1.54			[26]
	2	375.5	0.751	0.656	1.14	1hrs	1.4$	
	3	233.1	0.466	0.308	1.52			
SPIONs	1	985.3	1.861	1.232	1.51			Present
	2	843.4	1.711	0.902	1.89	35min	0.25$	Work
	3	664.3	1.513	0.871	1.73			
Mag. Beads	1	789.0	1.861	1.232	1.51			[28]
	2	622.4	1.711	0.902	1.89	45min	1.4$	
	3	584.3	1.513	0.871	1.73			

 # Deoxyribonucleic acid was diluted 1:100 in nuclease free water for spectrophotometric analysis

4.5 Agarose Gel Electrophoresis

The isolated MTB DNA analyzed through agarose gel electrophoresis experiment and discovered below the gel picture. Each MTB DNA shows with a single band at the same position (Fig 7). This result indicates that the eluted MTB DNA is very pure. Eluted DNA shows no smears or low molecular weight band were detected, indicating the absence of RNA contamination.

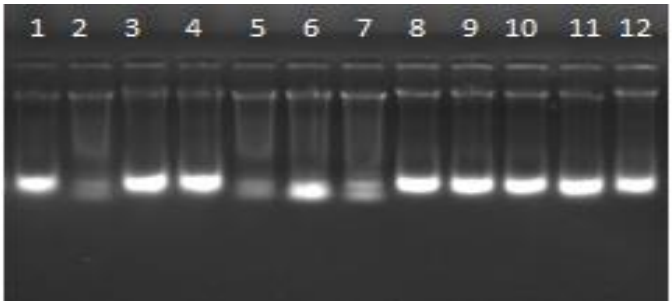

Fig 7. Electrophoresis of MTB DNA, Lane [1-3] chloroform-phenol, lane [4-6] commercial kit, lane [7-9] SPIONs and lane [10-12] magnetic beads.

4.6 Real time PCR (RT- PCR)

The sensitivity of all extracted DNA samples was performed on Real time Rotor Gene 2000/3000/6000, (Corbett Research Australia). MTBC- specific primers were used to amplify a target IS6110, the Accession number LC005454.1. An internal control (IC) plasmid genomic DNA used to detect PCR inhibition in the extracted MTB DNA [34,35]. All results are MTB positive confirmed by Real time PCR, Ct value shows for SPION (21.3) is lower as compared to the commercial kit,

indicates more amount of target nucleic acid is found. In real time assays, undergo 40 cycles of amplification.

5. Discussion

Recently Bandyopadhyay has previously reported magnetic nanoparticles and their use in the extraction of nucleic acids from *E.coli* [36]. Magnetic separation has established considerable attention in biomedical research because of its important properties of large surface/volume ratio, biocompatibility and low toxicity [37]. In the present study, we have developed SPION-mediated MTB extraction method and compared with conventional and commercial kit base method. The main advantage of our method is fast and cost effective, robust and accomplish in a single test tube. Commercial kit required USD 1.4 $ per sample. SPION mediated MTB DNA extraction serves this purpose only in USD 0.25 $ per sample, the details are shown in Table 1. The conventional chemical methods are time consuming and required trained person and the yield of DNA is very low. Therefore, based on the quantity and quality of DNA extracted, the SPION mediated DNA extraction method was better than conventional and commercial kit base method. XRD analysis confirms the crystallite size and phase purity of the SPION which shown in Fig.1. MTB DNA detection with SPION is possible because it provides a higher surface area the size of our particles is 9.7 nm. Magnetic particles less than about 30 nm shows superparamagnetism [38, 39]. FT-IR spectrum shows the peaks at 1626 cm^{-1} and 3407 cm^{-1} can be attributed to the stretching vibration of the hydroxyl groups on the surface of the SPION [40]. The FTIR study also suggests the formation of iron oxide nanoparticles. The properties of magnetic particles were verified by hysteresis loop measured by VSM. A typical plot of magnetization Vs. applied magnetic field (M-H Loop) of 298 K is shown in Fig.3. The saturation magnetization of SPION is 84emu/g^{-1}. This large saturation magnetization of SPION makes them very susceptible to magnetic fields and therefore makes the solid and liquid phases easily [41, 42]. The morphological study of SPION shows the formation of spherical and granular in shape and has strong magnetic dipole-dipole interaction, hence are attached strongly and form big clusters which shown in Fig.4. Purity and yield of the extracted DNAs were measured by UV-visible bio-spectrophotometer. The average A_{260} /A_{280} ratio and yield of SPION recovered DNA was approximately 1.8 to 2.0 indicates, least protein contamination, obtained by SPIONs. Thus, our study specifies that the organic methods yields lowest of MTB DNA quantity, whereas commercial magnetic bead-kit base method and our method yields good quality MTB DNA. We developed MTB DNA extraction method of positive sputum sample using SPION and its superiority has brought to focus for the first time through study. This method is even applicable for small sample volume and even low bacterial load. The rapid and efficient extraction of sputum sample is suitable for molecular identification techniques for real time PCR would be of excessive benefit. Magnetic isolation methods are simple, rapid, sensitive and environmentally friendly, are suitable for routine laboratory use, but also hold potential for building of automatic sputum DNA extraction

systems for several diagnostic purposes [43].

4. CONCLUSION

The emerging SPION- mediated extracting of MTB DNA technique is developed. This technique proves to be rapid, inexpensive, and robust accomplish in a single test tube. SPION-mediated MTB DNA extraction method produced pure and good quality DNA and are worthy for real-time PCR detection that amplifies successfully. In this study, we try to develop MTB DNA extraction from positive sputum sample using SPION method and its superiority has brought to focus for the first time through study. This method is even applicable for small sample volume and even low bacterial load. Looking towards the necessity and emergency, there is pressing need of development of a newer alternative method for detection of MTB DNA.

CONFLICT OF INTERESTS

The authors have declared that they have no conflict of interest.

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