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Nanotechnological Approaches for the Detection of Mycobacteria with Special References to *Mycobacterium avium* Subspecies *Paratuberculosis* (MAP)

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ABSTRACT

Tuberculosis, an infectious bacterial disease that affects the lungs is caused by *Mycobacterium tuberculosis* (MTB). It is the second most infectious disease after AIDS, which can affect both animals and humans. Johne's Disease (JD) or paratuberculosis caused by in intracellular bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an incurable wasting disease known to affect a large number of domestic animals and poses serious threat to livestock industries through huge economic losses. Conventional diagnostic methods like enzyme linked immunosorbat assay (ELISA), Polymerase Chain Reaction (PCR), cultural isolation are identification for use in MAP detection while sputum smear microscopy and PCR techniques remain the gold standards for TB detection despite advancement in pathogen detection most of these diagnostic methods are time consuming and have low efficacy and this become a heavy burden to developing and underdeveloped countries. When nanoscale particles are used as tags or labels, measuring the activity or presence of an analyte becomes faster, flexible and highly sensitive. These advantages nanomaterials possess, research have now focused their attention to nanotechnology based detection. Though research have shown these test to be more sensitive, less laboratorious and less time consuming, more needs to be done to introduce point of care diagnostics into the global market. This review highlights the prospects of nanotechnology based diagnostic tests as valuable alternative for rapid detection of this economically important pathogen with high accuracy and precision.

Key words: Paratuberculosis, *Mycobacterium tuberculosis*, mycobacteria, diagnosis, nano-technology, nano-diagnosis, nano-diagnostic

INTRODUCTION

Tuberculosis (TB) caused by various strains of mycobacteria are a multi-species disease occurring in 3 forms: bovine TB (*Mycobacterium bovis*), avian TB (*Mycobacterium avium*) and

human TB (*Mycobacterium tuberculosis*) and has high public health concerns (Radostits *et al*., 2000; Dhama *et al*., 2011; Raviglione and Krech, 2011; Perez-Lago *et al*., 2013; Singh *et al*., 2014; Verma *et al*., 2014), Of these, *M. tuberculosis* (MTB) still remains one of the most infectious disease in the modern world with an estimated 9 million new cases added every year (Shafiq *et al*., 2013). Owing to the limitations of current diagnostics and treatment methods available as well as emerging multi-drug resistant strains of *Mycobacteria* the goal of complete eradication of TB by 2050, continue to remain an ongoing challenge (Wang *et al*., 2013). Treatments involve long term, multidrug therapy, which ultimately leads to poor compliance of patients, causing multi drug resistant (MDR) TB for which only a few treatment options exists (Dhama *et al*., 2011; Tiwari *et al*., 2013). A key aspect that nowadays is being focused is the development of rapid diagnostic methods for TB, which would help to prevent and control the spread of the disease.

Johne's Disease (JD) or paratuberculosis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a non motile, aerobic, gram positive and acid fast pathogenic bacterium in the genus *Mycobacterium* is an economically important disease of ruminant animals that severely affects animal health and leads to heavy production losses to farmers and food industry owing to its high prevalence. The infection is characterized by weight loss and diarrhea leading to protein loosing entropy as a result of granulomatous inflammation of the intestine and lymph nodes (Sweeney, 2011). Evidences are continuously increasing on the zoonotic potential of MAP and many researchers have supported the association of MAP with Crohn's Disease (CD) in humans (Sartor, 2005). The CD is characterized as chronic, incurable, low-grade inflammation of the terminal ileum (Hermon-Taylor, 2009). Compelling evidence suggests that MAP can enter the human food chain through dairy supply and survives pasteurization (Chamberlin *et al*., 2001). The JD in animals, principally spreads through infected animals as they shed bacilli in their faces and milk, contaminating the environment (Sweeney *et al*., 1992). Therefore an early diagnosis of infected animals and their removal is important to stop the chain of infection spread and food security.

Diagnostic tests include isolation and identification of *Mycobacteria*/MAP in bacterial cultures along with serological and molecular tools to diagnose infected animals/individuals (Singh *et al*., 2014). Widely used methods for detection of MAP infection includes; enzyme linked immunosorbat assay (ELISA), Polymerase Chain Reaction (PCR) and culture while for TB include chest X ray, skin test and blood test (Singh *et al*., 2014). These techniques, though useful but suffer with issues of specificity or sensitivity or both and reproducibility varies between laboratories and also according to the disease stage in the host affected. Moreover, these techniques cannot be utilized as field and/or spot tests. Further, in developing countries where resources are few and the need for sophisticated, expensive instrumentation becomes a burden due to requirement of trained persons to perform the tests resulting in higher cost for diagnosis, there is need to develop and adapt appropriate rapid diagnostic tests utilizing the advances in science and technology. From these perspectives, the applications of nanotechnology based diagnosis can offer quick and efficient alternative methods for detection of mycobacterial diseases. This review highlights prospects of the advances in the nanotechnology based approaches that can offer better solutions for diagnosis of various mycobacterial infections.

Nanodiagnostic approaches: The recent progress in the field of nanotechnology has showed its wide potential and various beneficial applications in biomedicine, biotechnology, human and animal health, including saliently the highly useful nanodiagnostics, nanomedicines and nano drug

delivery mechanisms (Jain, 2007; Dhama *et al*., 2008; Chakravarthi and Balaji, 2010; Boisseau and Loubaton, 2011; Manuja *et al*., 2012; Dilbaghi *et al*., 2013; Num and Useh, 2013). Nanoparticles can be used for the immobilization of particular ligands on their surfaces as a result; they have emerged as a perfect candidate for the sensitive disease diagnosis (Kawadkar *et al*., 2011; Dhama *et al*., 2008; Num and Useh, 2013). The small size (1-100 nm) and large surface area of nanomaterials results in enhanced properties such as surface reactivity, quantum confinement effects, electrical conductivity and magnetic properties. Nanodiagnostics have redefined the standards for molecular diagnostics, triggering the development of new approaches in biomolecular recognition and analytical systems, where the most promising approaches include nanoparticles (NPs), nanotubes, nanopores and nanocantilever technologies (Jain, 2007; Dhama *et al*., 2008; Num and Useh, 2013; Baptista *et al*., 2008). Early detection of Johne's disease would help in isolating the infected animals, preventing contamination of the rest of the herd. Alongside speed and sensitivity, affordability, robustness and reproducibility are important factors that contribute to the advantages of nano based diagnostics usage (Kaittanis *et al*., 2010). Nanotechnology based approach for the diagnosis of MAP has been the focus of research as this provides an alternative for the identification of molecular targets *in vitro* and *in vivo*, that require only a small amount of samples, lesser tie for preparation and good sensitivity along with delivering quick diagnosis (Rosi and Miskin, 2005).

An overview of the nanotechnology based approaches for diagnosis of mycobacteria is presented in Table 1.

Noble metal nanoparticles: For smart sensing devices, gold nanoparticles (GNPs) are one of the most suited nanomaterials. Due to their unique properties they are best choice nanomaterials in various multidisciplinary research (Mieszawska *et al*., 2013). The compatibility of GNPs is excellent with antibody or antigen and other biomolecules; moreover, GNPs do not affect the functional activity even after immobilization. The antibody antigen reaction is accelerated by the surface functionalization of gold nanoparticles, thereby amplifying immunoassay signalling (Mieszawska *et al*., 2013). The use of thiol-linked single-stranded DNA modified gold nanoparticles (herein referred to as AuNP-probes) for the colorimetric detection of DNA targets represents an inexpensive and easy to perform alternative to PCR, fluorescence or radioactivity based assays (Elghanian *et al*., 1997).

Table 1: Summary of the nanotechnology based approaches for diagnosis of mycobacteria

Technology	Description	Application	References
Noble metal NPs	Detection based on colometric changes that can be visualized by the human eye	Specific detection of MAP and *M. tuberculosis*	Mieszawska *et al*. (2013) Elghanian *et al*. (1997)
Quantum dots	Detection based on fluorescence	Specific detection of MAP and *M. tuberculosis*	Gazouli *et al*. (2010)
Surface enhanced Raman scattering (SERS)	Detection based on SERS signals produced when antibodies bind to Raman Label antigens	Specific detection of MAP	Yakes *et al*. (2008)
Electrochemical devices	Detection is based on electrical conductance	• Specific detection of *M. tuberculosis* by nanostructured zinc oxide (nsZnO) films • Rapid and specific detection of MAP by polyanalinie based electrochemical sensors	Gazouli *et al*. (2010) Okafor *et al*. (2008)) Kumanan *et al*. (2009)
Silica nanoparticles	Large number of fluorophore molecules inside the silica amplify the fluorescence, making otherwise undetectable analytes visible for detection	Specific detection of *M. tuberculosis*	Qin *et al*. (2008)

The first application of AuNP in the diagnosis of TB was done in 2006 (Baptista *et al*., 2006). In this method, PCR was followed by colormetric detection that uses gold nanoparticles for the direct detection of *M. tuberculosis*. The set up consists of functionalized gold nanoparticle probe, which consists of a specific oligonucleotide derived from the gene sequence of the *M. tuberculosis* RNA polymerase subunit. At 526 nm the nanoprobe solution is pink which turns purple due to nanoprobe aggregation at a high NaCl concentration due to lack of complementary DNA. So, when the probe hybridizes with *M. tuberculosis* DNA no aggregation occurs and the solution remains pink. The sensor proved to be accurate when compared to other methods like INNOLiPA-Rif-TB, which gave 100% concordance (Baptista *et al*., 2006). The design was further modified by Liandris (Liandris *et al*., 2009), that sought the aggregation of gold nanoprobes by increasing acid concentration instead of salt concentration. The test was proved to be sensitive and can be simply visualized for detection.

Hussain *et al*. (2013) reported a prototype based on rapid nano-gold assay for detection of *M. tuberculosis* complex (MTBC). A 16s rDNA regions of *M. tuberculosis* were amplified by PCR and amplicons were identified using genus and species-specific oligotargeters and gold nanoparticles. With a turnaround time of 1 h, the prototype when compared with automated liquid culture system (BACTEC™ MGIT™) and semi-nested PCR was shown to be simple, specific, sensitive and can replace PCR based detection.

A colorimetric method has been developed that depends on the use of gold nanoparticles for quick and precise detection of mycobacterial species, eliminating the requirement for DNA amplification (Liandris *et al*., 2009). The results can be obtained similar to that of TB detection designed by Liandris by comparing the gold nanoparticles probe before and after aggregation induced by acid. The aggregation is prevented by the presence of a complementary target and as a result the solution remains pink, whereas in the contrary situation the solution turns purple. The AuNP-probe solution exhibits a pink colour because of surface plasmon resonance at an absorbance peak of ~525 nm. The acidic environment after the addition of HCl enhanced precipitation of AuNP probe in the absence of a target DNA sequence leading to a change of colour from pink to purple (indicating an absorbance peak shift toward the longer wavelength). In the event of specific probe hybridization to a target sequence (i.e. mycobacterial DNA), no AuNP-probe aggregation occurs and the solution remains pink. Thus, the reading of the method described herein can be obtained by visual and spectrophotometric comparison of the solutions before and after acid induced Au nanoprobe aggregation. The proposed approach is considered an easy low cost assay that takes approximately 15 min per sample and carried out in a single tube reducing contamination and allows simultaneously multiple testing of samples. The assay was also found to be highly specific and reliable with a minimum mycobacterial DNA requirement.

Karthik *et al*. (2011) developed a dipstick immunoassay for MAP diagnosis using gold nanoparticles, which was considered to be highly specific and sensitive (Karthik *et al*., 2011). In this assay MAP protoplasmic antigen coated on gold nanoparticles binds with anti MAP rabbit antibodies immobilized on nitrocellulose membranes. The binding could be visually detected due to the development of red colour formed by the gold nanoprticles on the membrane. The immunoassay test was considered highly specific and when efficacy was very high when compared to tests like agar gel immunodiffusion (AGID) and absorbed ELISA.

Quantum dots: Quantum dots are nanocrystals of a semiconductor material, ranging from the size of 2-10 nm (Smith *et al*., 2008). Due to the high surface to volume ratio of these particles, they

exhibit fluorescent properties. Broad absorption spectra, narrow emission spectra, slow excited-state decay rates, broad absorption cross-sections and being highly photostable are some of the advantages quantum dots posses over other fluorescence based methods (Rizvi *et al*., 2010). Gazouli *et al*. (2010) developed a method for the rapid detection of mycobacterial DNA using fluorescence quantum dots and magnetic beads eliminating the need for DNA amplification (Gazouli *et al*., 2010). Cadmium selenite quantum dots conjugated with streptavidin and species-specific probes produced the fluorescent signals while magnetic beads conjugated with streptavidin and genus-specific probes were used to concentrate the DNA targets. Mycobacterial DNA was targeted through a sandwich hybridization reaction which consisted of 2 biotinylated oligonucleotide probes that would recognize and detect the target DNA. The accuracy of the proposed method in comparison to real time PCR was found to be 70-90%, depending on the clinical sample.

Silica nanoparticles: Mesoporous silica has application in various fields like imaging, drug delivery and biosensors (Slowing *et al*., 2007). The use of silica nanoparticles in *M. tuberculosis* detection was explored by Qin *et al*. (2008), who designed a method for the detection of *M. tuberculosis* by fluorescent nanoparticle-based indirect immunofluorescence microscopy (Qin *et al*., 2008). The setup consists of nucleic acid dye SYBR Green I which was stained on bioconjugated fluorescent silica nanoparticles. This assay takes only 2 h and is considered a promising method for the rapid detection of *M. tuberculosis*. There are no data so far where silica nanoparticles were used for the detection of MAP.

SURFACE ENHANCED RAMAN SCATTERING

A novel sandwich immunoassay for MAP detection based on Surface-Enhanced Raman scattering (SERS) has been proposed. Assay consists of an immobilized layer of monoclonal antibodies that targets surface antigens on MAP followed by labelling the antigen with extrinsic Raman labels that specifically binds to capture antigens and produce large SERS signals which can be quantified. The detection of the assay was estimated to be 5000-10000 MAP bacilli mL^{-1} depending on the sample type. Specificity, sensitivity and speed are some of the advantages the assay posses over conventional methods (Yakes *et al*., 2008).

Biosensors: A biosensor is an analytical system intended for detection or quantification of the presences or absences of any biological analyte by integrating a bio-recognition element a transduction system and electronic devices like amplifiers and display unit (Van den Hurk *et al*., 2015). In 1997 an electrochemical biosensor for *M. tuberculosis* detection was developed by Wang *et al*. (1997). The biosensor is based on the determination of short sequences of *M. tuberculosis* DNA. A carbon paste transducer was modified with oligonucleotide probes and hybridized with the direct repeat region of the MTB DNA which was ultimately transduced by chronopotentiometry. In Prabhakar *et al*. (2008) developed a biosensor that consisted of a probe specific to the rRNA spacer region of MTB that were immobilized onto a polypyrrole-polyvinylsulphonate film by covalent bonding and was then was deposited electro-chemically onto Indium-Tin-Oxide (ITO) glass to detect complementary targets.

Kim *et al*. (2012) developed an immunosensor that specifically detects mycobacterial cells by a combination of electric fields, streaming flow and immuno-affinity binding (Kim *et al*., 2012). This method involves the novel mechanisms of concentrating bacteria, thus facilitating the quick

detection of *M. tuberculosis* cells in sputum samples, avoiding the conventional method of smear microscopy, which is less sensitive. The simple "Dipping and withdrawal" mechanism of the sensor also enables the screening to be done by minimal trained personnels reducing the cost of operations ideal for point of care screenings.

The first biosensor for MAP was developed by Okafor *et al*. (2008) that detects the antibodies against MAP. In principle the biosensor consist of 4 membrane namely; application, conjugate, capture and absorption membranes. Once a sample is applied to the application membrane it moves through the rest of the chamber through capillary action. On reaching the conjugate membrane where MAP and non MAP IgG bind to polyaniline/anti-bovine IgG, it forms a complex which is captured in the capture membrane. The MAP antigen immobilized on the capture membrane captures the MAP IgG while the non MAP IgG flows to the absorption membrane. As more MAP IgG gets captured the Pani-AB/IgG*-IgG complex forms a bridge between the silver electrodes present on either side of the capture membrane causing an electrical conductance which is recorded. On testing the biosensor with known JD positive and negative serum it showed within 2 min MAP IgG could be detected.

A year later a simple membrane strip lateral flow biosensor assay was developed by Kumanan *et al*. (2009) that use a membrane flow-through system accompanied by an immobilized DNA probe to hybridize to the target. When a second DNA probe coupled to liposomes encapsulating sulforhodamine B (dye) hybridizes with a target sequence a signal amplification is produced. Liposomes confined in the detection zone can be either read with a reflectometer or quantified visually. The test concluded that the method was sensitive and was able to detect 10 organisms per 100 mg of feces (Kumanan *et al*., 2009).

Karthik *et al*. (2013) developed a bioelectronics sensor for the diagnosis of paratuberculosis in goats (Karthik *et al*., 2013). It consists of an electrically active polyaniline coated magnetic nanoparticles (EAPM) which was fabricated with rabbit anti-goat IgG. Antibodies against MAP were detected by the protoplasmic antigen immobilized on a capture pad. The detection of the antibody by the anti-goat IgG bound to EAPM was established by an electric circuit that bridged the nanopartciles with the silver electrodes present of on either side of the capture membrane. The electrical conductance was measured between the electrodes as a direct charge transfer. When the biosensor was compared to other standard tests like absorbed ELISA, AGID (Agarose Gel Immunodiffusion) nested PCR and Johnin test it was found that though bisosensor had low sensitive but was rapid and simple for field work (spot test).

Though studies are very preliminary but the results of MAP biosensors are very promising in terms of use as field spot test. Hence, further research is demanded in this area.

CONCLUSION

Over the last few decades, tremendous advancement has been made in nanotechnology in various fields, especially medicine, by the introduction of new approaches to molecular detection and treatment. Tuberculosis and paratuberculosis are both caused by the pathogenic bacteria's of the Mycobacteriaceae family. The gold standard methods used for detection of theses diseases have many disadvantages of being less sensitive, time consuming and require skilled professionals. While research for nanotechnology based detection of tuberculosis has reached advanced stages, for paratuberculosis the research is still at infancy. The evident advantages of nanodiagnostics involve their ability to produce results in a short time frame, along with the high specificity and sensitivity when compared to conventional method of detection like microscopy, culture and ELISA and amplification of nucleic acid based detection. Nonetheless, very few nanodiagnostic methods

have been translated into the clinical settings for TB and none so far for paratuberculosis. Since the pathogens involved, spread the disease by contaminating the environment, detection at the earliest is most important for containing the spread of the disease, hence future trends involve the use of nanodiagnostics in biochip technology scale down to the nanoscale range for point-of-care diagnostics with a sample-in answer-out approach which eliminates human error, thus facilitating their use by non-specialized personnel.

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