INTRODUCTION

Microscopy is currently the most widely used tool for the diagnosis of TB. Although lacking sensitivity, smear microscopy is inexpensive, relatively simple to perform and a rapid diagnostic test \cite{1,2} that can be performed close to the point-of-care (POC). Culturing *Mycobacterium tuberculosis* (*M. tuberculosis*) is a much more sensitive means of diagnosis than sputum smear microscopy and is the reference standard for diagnosis of *M. tuberculosis* \cite{3}. However, *M. tuberculosis* is a slow growing organism \cite{2} and culture of clinical specimens typically takes from one to six weeks, depending on the bacterial load in the sample. Molecular methods (such as nucleic acid amplification (NAA) tests) have a rapid turnaround time and are thus attractive diagnostic tools. However, NAA tests often lack sensitivity. Theoretically, NAA tests require small quantities of bacteria \cite{4,5} and until recently (with the introduction of the Xpert MTB/RIF test (Cepheid) \cite{6}) have not been widely adopted \cite{7}. Optimal specimen processing protocols are key for the sensitive detection of *M. tuberculosis* bacteria by NAA testing, and require extraction, concentration and purification steps.

Specimen processing prior to culture and NAA testing for TB

Specimen processing is a critical and underestimated step when attempting to isolate *M. tuberculosis* from sputum \cite{8,9}. Development of optimal sample processing is not sufficiently prioritised although there is an urgent need for it to go hand-in-hand with novel detection systems \cite{9}.

Although *M. tuberculosis* has the ability to infect a range of organs, the most common form of tuberculosis (TB) in adults is pulmonary TB \cite{9}, with sputum the specimen most frequently collected for diagnosis. Inefficient specimen processing will hamper the release of *M. tuberculosis* bacteria and or DNA from specimens, which will result in poor sensitivity of diagnostic tests. This is particularly relevant for patients with paucibacillary TB, which is common in children and in adults co-infected with HIV.
Theoretically, NAA tests require small quantities of bacteria, however, in practice they generally have suboptimal sensitivity in smear negative sputum specimens, which may, in part be due to the relative inefficiency of the sputum processing protocols used.

**Sputum**

Sputum is a mixture of mucus with other endogenous or exogenous components which may include transudated and exudated fluids, a range of local and migrated cells, microorganisms, necrotic tissues or cells, aspirated vomitus and other foreign particles.

Respiratory mucus forms part of the innate immune system. It is divided into two phases viz, a sol (fluid) and a gel phase. Respiratory mucus is made up of water, ions, proteins, lipids, enzymes, immunoglobulins and large glycoproteins of varying sizes. These glycoproteins are called mucins.

Mucins form a dense, protective barrier preventing bacterial attachment to cells lining the respiratory tract. Mucus traps bacteria and debris which are naturally moved out of the respiratory tract by ciliary clearance.

There are approximately 20 genes involved in human mucin expression. The glycoproteins encoded by the mucin genes are divided into three major families namely; membrane associated mucins (MUC1, MUC4, MUC11, MUC13, MUC15 and MUC20), non-gel forming mucins (MUC7) and gel-forming mucins (MUC5AC and MUC5B, MUC2, MUC8 and MUC19). The gel forming mucins, MUC5AC and MUC5B, comprise 79% of normal human respiratory secretions. The MUC5AC gene is expressed in limited to the goblet cells of the lung while MUC5B is expressed in the bronchiolar epithelium and submucosal glands.

In order for efficient ciliary clearance to occur, mucus must possess high elastic recoil together with low viscosity. However, in the presence of disease (such as cystic fibrosis and bronchitis), alterations of the terminal glycosylations of mucins occur resulting in a physical change in the composition and nature of mucus.

**SPUTUM PROCESSING**

Sputum processing prior to routine microbiological testing

Sputum processing in the diagnostic laboratory for non-tuberculosis specimens usually includes Gram stain and direct inoculation onto agar. Sputum processing for mycobacterial culture however, involves liquefaction, decontamination, neutralisation and concentration. During liquefaction a mucolytic agent is added to sputum to release bacteria that may be trapped within the complex sputum network. This mucolytic is often also a decontaminant that kills contaminating microorganisms that might affect downstream mycobacterial culture. Petroff’s method which employs 4% sodium hydroxide (NaOH) was previously used for liquefaction and decontamination but this concentration of NaOH is extremely harsh on the tubercle bacilli. It was
shown that decreasing the concentration of NaOH and adding a digestant, N-Acetyl-L-Cysteine (NALC), increases the likelihood of isolating *M. tuberculosis*. Ratnam et al. showed NaOH at a 1.5% concentration to be sufficient to prevent bacterial overgrowth during culture for *M. tuberculosis* for most sputum samples [56, 58]. Following liquefaction and decontamination, a neutralising substance (e.g., phosphate buffer (pH 6.8) when using an alkaline digest) [59] is added in a timely manner in order to stop the decontamination process, thereby reducing the impact of the mucolytic on the viability of mycobacteria. Neutralisation is typically followed by concentrating the released bacteria in a cell pellet. This is usually achieved by centrifugation.

**Sputum processing prior to NAA testing**

Sputum processing for NAA tests typically involves sputum liquefaction (often with use of a mucolytic agent); *M. tuberculosis* cell lysis (mechanical and/or chemical lysis may be utilised); followed by DNA purification in order to concentrate DNA and remove potential PCR inhibitors.

If both culture and NAA testing are required on a specimen, sputum processing prior to NAA testing may follow similar processes to those used prior to mycobacterial culture (in which case the pellet can be split between culture and NAA testing) [60]. Pathak et al. has shown long-term storage of sputa at -20°C subsequent to NALC treatment results in greater yield of *M. tuberculosis* DNA [61].

If culture is not required, as cell viability is not a prerequisite for NAA tests, more stringent processing can be employed in order to kill mycobacteria (as a biosafety precaution) and maximize release of most of the bacteria from the sputum. For example, sputum can be collected directly into media, which preserve nucleic acid; however, these have generally not been well-validated for *M. tuberculosis*. Examples include PrimeStore® Molecular Transport Medium (PSMTM; Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) [62], cetylpyridinium chloride (CPC) [63] and Universal sample processing solution (USP) [64]. Primrose has some mycobacterial activity [65, 66]. The Universal sample processing (USP) solution has not been used as transport medium; however, it contains 4-6 M guanidinium hydrochloride (which may aid in cell lysis), 50 mM Tris/Cl (which maintains the pH), 25 mM EDTA (a chelating agent), 0.5% Sarkosyl (anionic detergent that disrupts cell membranes) and 0.1 M β-mercaptoethanol (which may reduce cysteine residues). Solutions containing Guanidinium have been shown to preserve nucleic acids at room temperature for prolonged periods of time [67] hence the USP solution may be adapted for use as a transport medium. CPC can also be utilised as a transport medium [63] and is compatible with the Xpert MTB/RIF assay [68] whereas the USP solution has been shown to be compatible with DNA as well as RNA isolation [64].

**Chemical and mechanical sputum liquefaction**

Sputa can be liquefied chemically (by the use of mucolytic agents) or mechanically.

**Chemical liquefaction of sputum**

Mucolytic agents are used to liquefy/digest sputum. Some mucolytics are administered orally (oral alpha-chymotrypsin) to improve mucociliary clearance in diseased individuals. Others are employed in vitro (NALC-NaOH) for microbiological investigations. Liquefaction results in a change in the biophysical properties of sputum usually by reduction of mucin molecules, fibrin, F-actin and DNA [69]. For mucin, this involves the separation of the intermolecular hydrogen bonds which link mucin molecules, which in turn results in a reduction of entanglement points and hence contributes to a decrease in viscosity [70].

There are different types of mucolytics, namely, classical and peptide mucolytics. Classical mucolytics act on the primary network, by digesting bonds linking the mucin network. Peptide mucolytics act on the secondary network, which comprises cellular debris, F-actin and DNA [69].

Heterogeneity within and between individual sputum samples is due to the intricate structure of mucus and the variable nature of the underlying pathology [71]. Therefore mucolytic agents may not be effective on all sputum samples as they may act on targets not present in a specific sputum specimen or as physical or chemical barriers may prevent the agent from accessing its target. As an example, Deoxyribonuclease (DNase) does not act on the mucoprotein gel, which may be present in certain sputum samples, and may therefore not be an efficient mucolytic for all sputum samples [41].

When samples are being processed for culture, the liquefaction process usually occurs concurrently with sputum decontamination. The liquefaction and/or decontaminating agent is typically added to the sputum specimen followed by incubation at room temperature for 15 - 20 minutes. Some investigators have suggested incubating viscous specimens at 37 °C [72, 73]. It is important to note that some liquefaction and decontamination procedures (Zephiran-Trisodium Phosphate, Sodium Lauryl Sulphate, Cetylpyridinium chloride or other quaternary ammonium compounds) are only compatible with egg-based culture media and cannot be used in conjunction with the Mycobacteria Growth Indicator Tube (MGIT™) system [59].

Currently the most widely used method for liquefaction and decontamination of sputum is that described by Kent and Kubica (1985) [74]. This method involves the use of NALC (0.5% final conc.) and NaOH (2% final conc.) together with sodium citrate (1.45% final conc.) [59].

The extent to which sputum liquefaction occurs can be measured by means of a viscometer. Examples of viscometers used
in sputum liquefaction studies include the Consisto-viscometer [75]; the Brookfield viscometer [76] and the sputum consistometer [77]. Another means of determining sputum liquefaction is to take note of the liquid portion and the pellet following centrifugation. Smaller, more compact pellets with an increase in the fluid component may signify more efficient liquefaction.

**Types of mucolytics**

A summary of mucolytics is presented in **Table 1**. Even though a variety of liquefaction agents exists, NALC/NaOH and dithiothreitol (DTT) are the mucolytics that are most widely used for sputum liquefaction prior to *M. tuberculosis* culture and NAA testing. Although potent, these mucolytics are classical mucolytic agents which act on mucin and not on the secondary network present in sputum which contributes to sputum viscosity. Efficient reduction of the primary and secondary network present in sputum may theoretically result in greater sensitivity of *M. tuberculosis* culture and NAA tests.

**Table 1.** Mucolytic agents.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mucolytic</th>
<th>Possible mechanism of action</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>NALC</td>
<td>Severs disulphide bonds</td>
<td>Reduces sputum viscosity</td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>Severs disulphide bonds</td>
<td>Reduces sputum elasticity more than 90%</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin</td>
<td>Severs disulphide bonds</td>
<td>Reduces sputum viscoelasticity</td>
<td>[79]</td>
</tr>
<tr>
<td>Peptide</td>
<td>DNase</td>
<td>Digests deoxyribonucleic acid/protein fibres</td>
<td>Reduction in viscosity and elasticity</td>
<td>[40, 80, 81]</td>
</tr>
<tr>
<td></td>
<td>Gelsolin</td>
<td>Digests F-actin</td>
<td>Reduces sputum elasticity, viscosity and cohesivity; Drastic reduction in elasticity (77.3%) and viscosity (80.4%)</td>
<td>[32, 37, 69]</td>
</tr>
<tr>
<td></td>
<td>Anionic (Poly) amino acids</td>
<td>Dissolves histones which form DNA and F-actin bundles in sputa, Enhances the activity of Dnase 1</td>
<td>Decrease in sputum viscosity</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Thymosin b4</td>
<td>Digests F-actin</td>
<td>Improve mucociliary clearance in vitro in combination with Dnase 1</td>
<td>[37, 38]</td>
</tr>
<tr>
<td></td>
<td>UFH</td>
<td>Digests DNA and F-actin bundles, Enhances the activity of Dnase</td>
<td>Decreases elasticity but not viscosity</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>Chymotrypsin, trypsin, pancreatin</td>
<td>Breaks down cellular debris</td>
<td>Reduction in sputum viscosity and improvement in expectoration in vivo</td>
<td>[82, 83]</td>
</tr>
<tr>
<td></td>
<td>NaOCl</td>
<td>Breaks down cellular debris</td>
<td>Reduction in cellular debris on smears on microscope slides compared to direct smears</td>
<td>[84, 85]</td>
</tr>
<tr>
<td></td>
<td>USP</td>
<td>May aid in cell lysis, disrupts cell membranes, may reduce cysteine residues</td>
<td>Minimal backgrounds on slides prepared compared to direct smears</td>
<td>[64, 72]</td>
</tr>
<tr>
<td>Unknown</td>
<td>Modified Jungmann’s method</td>
<td>Breaks down cellular debris</td>
<td>Produces a ZN stain <em>free from artefacts</em></td>
<td>[86]</td>
</tr>
<tr>
<td>classification</td>
<td>Chitin</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-with hexa-fluoroisopropanol</td>
<td>Digests sputum more rapidly than NALC in 2% NaOH and NaOCl</td>
<td></td>
<td>[87, 88]</td>
</tr>
<tr>
<td></td>
<td>-with sulphuric acid</td>
<td>Digests sputum more rapidly than 4% NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPC</td>
<td>Unknown</td>
<td>Digestant, decontaminant and preservative for up to 20 days at room temperature</td>
<td>[63, 89-92]</td>
</tr>
<tr>
<td></td>
<td>iodated</td>
<td>unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>compounds</td>
<td>Xpert MTB/RIF sample reagent</td>
<td>Changes the &quot;potential protein substrate in sputum&quot;</td>
<td>induces enzymatic proteolysis</td>
<td>[93]</td>
</tr>
<tr>
<td></td>
<td>CPC – cetylpyridinium chloride; DNA – deoxyribonucleic acid; DNase – deoxyribonuclease; DTT – dithiothreitol; F-actin – filamentous actin; NALC – N-acetyl-cycteine; NaOCl – sodium hypochlorite; NaOH – sodium hydroxide; UFH – unfractionated heparin; USP – universal sample processing solution; ZN – Ziehl-Neelsen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Classical mucolytics**

Classical mucolytic agents, NALC, DTT and Thioreredoxin (Trx), break the disulphide bonds that link the mucin monomers (Table 1). *In vitro* studies show NALC to be a potent mucolytic agent when used in conjunction with NaOH [87]. Although NALC is currently the most widely used mucolytic agent in sputum processing, there is contradictory evidence on its utility. A study by Lorian and Lacasse demonstrated that 0.5% NALC plus 2% NaOH liquefies sputa similarly to 2% NaOH [95]. However, Kubica et al. found that the addition of NALC to NaOH enhances liquefaction. Dippy and Davis showed a significant reduction in sputum viscosity
with the use of 20% NALC (without the addition of NaOH). Digestion and decontamination described by Kent and Kubica includes the addition of 0.2% NALC/4% NaOH mucolytic to the sputum specimen at a 1:1 ratio. The specimen/mucolytic suspension is mixed (by inverting the tube several times or briefly vortexing) and incubated at room temperature for 15 minutes. Subsequent to incubation, a neutralising agent (sterile distilled water or phosphate buffer (pH 6.8)) is added to stop the action of NaOH on the tubercle bacilli. The suspension is thereafter centrifuged at 3000 xg for 15 minutes. The supernatant is removed and discarded safely and appropriately and the sediment is resuspended in phosphate buffer (pH 6.8).

In its native form NALC is a white crystalline powder which has a shelf life of up to 3 years if refrigerated. However a major disadvantage of NALC is that once solubilised, mucolytic activity is lost after 24 hours [59,74,96] resulting in the need for daily reconstitution of NALC/NaOH solution [97]. In addition, high concentrations of NALC and NaOH may affect detection of growing M. tuberculosis in the MGIT™ liquid culture detection system [99].

Sputum liquefaction using DTT has been shown to be more effective than NALC75, 79 even at low concentrations (0.1 M DTT vs. 1.2 M NALC) [79]. DTT has the ability to effectively liquefy sputum, showing a >90% reduction in sputum elasticity [32]. Another study which compared sputum processing prior to cytomorphic examination for diagnosing lung cancer, found sputum processing with DTT (0.2% final concentration) to be superior to NALC (1% final concentration), with regards to cellularity (P < 0.0001) [98].

Sputa from cystic fibrosis patients are thick and viscous and difficult to homogenise solely by chemical means [99]. In vitro studies have demonstrated that Thioredoxin (Trx) is able to liquefy thick, purulent spu from cystic fibrosis patients more efficiently than NALC and DTT [79]. Liquefaction was noted using Trx concentrations as low as 1 µM with maximum efficacy at 30 µM. Both DTT and Trx have greater muco-active capacities than NALC. The authors suggested that this may be due to Trx and DTT being diholis (having 2 redox-active cysteine residues) whereas NALC is a monothiol [79].

**Peptide mucolytics**

Peptide mucolytics act on the secondary network present in sputum. The secondary network is composed of cellular debris, F-actin and/or DNA. Various peptide mucolytics have been reported and are listed in Table 1.

DTNase liquefies purulent sputum by digesting DNA/DNP fibres that contribute to sputum viscosity [40]. These fibres are formed by extracellular DNA/DNP from cells undergoing degeneration. The DNA present in intact cells is not affected by DNase digestion. However, when processing sputa for NAA tests, caution should be taken when employing DNase, as cell free DNA of the target bacteria under investigation may be present in the specimen. DNase does not affect the viscosity of mucoid specimens as the enzyme has no effect on the mucoprotein gel [41].

The current literature on the mechanism of action of sodium hypochlorite (NaOCl, household bleach) on sputum is inconclusive. However, studies have demonstrated a reduction in debris visualised on smear microscopy when using NaOCl [84,85] as well as associated increased sensitivities when compared with direct smears [84,85,100]. This may be an indication that NaOCl acts as a peptide mucolytic, breaking down the secondary network formed in sputum. NaOCl has a dual function in sputum processing; it can be used as a digestive and a decontaminant (having the ability to kill contaminating bacteria as well as M. tuberculosis) [85].

A 2.5% (final concentration) NaOCl solution is able to decontaminate and digest purulent sputa within 30 minutes [85]. Although 2.5% NaOCl kills bacteria, including M. tuberculosis, M. tuberculosis does not lose its “acid-fastness”. However, direct treatment of slides with 5% NaOCl substantially reduced the number of bacilli visualized on smear microscopy [101]. This is probably due to exposure of a thin layer of specimen to 5% NaOCl as compared to processing an entire specimen.

**Unclassified mucolytics**

There are several mucolytics that have not been classified as classical nor peptide mucolytics (Table 1). The mechanisms of action of these mucolytics are mostly unknown. However, the Modified Jungmann’s method (ferrous sulphate, sulphuric acid and hydrogen peroxide) aids in breaking down cellular debris in the sputum and iodated compounds are said to, together with a protease (such as trypsin), change the nature of sputum proteins by inducing proteolysis. Data on the use of these mucolytics are scarce.

**Commercial/proprietary mucolytics**

The Xpert MTB/RIF sample reagent contains NaOH and isopropanol (concentrations are not known, proprietary information). Sputa collected from patients are treated with sample reagent for 15 minutes. The Xpert MTB/RIF sample reagent efficiently digests sputum and efficiently reduces viability of M. tuberculosis by 8 log [94].

**Mechanical liquefaction of sputa**

Mechanical digestion is another means of liquefying sputum and is often used in conjunction with chemical digestion. Sputa can be liquefied by vortexing with the aid of glass beads or by ultrasonication. This can be done in the presence or absence of a mucolytic agent.

The tendency of mycobacteria to clump within specimens might affect sensitivity when samples are split for routine
<table>
<thead>
<tr>
<th>Mucolytic</th>
<th>Final Conc. (%)</th>
<th>Ratio</th>
<th>Mechanical</th>
<th>Other</th>
<th>Microbial investigation</th>
<th>Sample fresh/frozen</th>
<th>Sample splitting</th>
<th>In-house/commercial PCR</th>
<th>Target/ test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DTT</td>
<td>0.05</td>
<td>1:1</td>
<td>Sonication</td>
<td>boiling</td>
<td>Microscopy, culture, PCR</td>
<td>fresh</td>
<td>Microscopy - unknown, Culture - 200 µl, In-house PCR - sediment (1000 µl), Amplicor - 100 µl</td>
<td>In-house IS6110</td>
<td>90.76</td>
<td>97.6</td>
<td>[111]</td>
</tr>
<tr>
<td>2</td>
<td>DTT</td>
<td>0.05</td>
<td>1:1</td>
<td>unknown</td>
<td>According to manufacturer's instructions</td>
<td>fresh</td>
<td></td>
<td>Commercial Amplicor</td>
<td>76.92</td>
<td>98.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>DTT</td>
<td>unknown</td>
<td>unknown</td>
<td>Sonication</td>
<td>Siliconised glass beads, proteinase K</td>
<td>Microscopy, culture, PCR</td>
<td>Fresh, however, samples prepared for PCR were frozen subsequent to processing</td>
<td>Microscopy - negligible, culture - 0.5 ml, PCR - 0.5 ml, gas chromatography-mass spectrometry - 1 ml</td>
<td>In-house IS6110</td>
<td>95</td>
<td>93</td>
<td>[109]</td>
</tr>
<tr>
<td>4</td>
<td>NALC-NaOH</td>
<td>0.5-1</td>
<td>1:1</td>
<td>Sonication</td>
<td>SDS-Tris-HCl, zirconia/silica beads, Nuclisens lysis buffer</td>
<td>Microscopy, culture, PCR</td>
<td>frozen</td>
<td>Microscopy - unknown, Culture - unknown, In-house PCR - unknown</td>
<td>In-house SecA1</td>
<td>97</td>
<td>97</td>
<td>[108]</td>
</tr>
<tr>
<td>5</td>
<td>NALC-NaOH</td>
<td>unknown</td>
<td>1:1</td>
<td>unknown</td>
<td>According to manufacturer's instructions</td>
<td>Microscopy, culture, PCR</td>
<td>frozen following decontamination, microscopy and culture</td>
<td>Microscopy - unknown, Culture - 500 µl, BD ProbeTec - 250 µl, Amplicor - 100 µl</td>
<td>Commercial Amplicor</td>
<td>89.5</td>
<td>87.5a 100b</td>
<td>[112]</td>
</tr>
<tr>
<td>6</td>
<td>NALC-NaOH</td>
<td>unknown</td>
<td>1:1</td>
<td>FastPrep</td>
<td>lyosyser - heat @ 105 °C</td>
<td>Microscopy, culture, PCR</td>
<td>frozen</td>
<td>Microscopy - unknown, Culture and Microscopy - 2/3 sediment, In-house PCR - 1/6 sediment, Gen-Probe AMTD - 1/6 sediment</td>
<td>In-house SecA1</td>
<td>24a 20b</td>
<td>95a 99b</td>
<td>[113]</td>
</tr>
<tr>
<td>7</td>
<td>NALC-NaOH</td>
<td>0.5-1</td>
<td>1:1</td>
<td>Sonication</td>
<td>NALC-SDS-Tris-HCl, zirconia/silica beads, Nuclisens lysis buffer</td>
<td>Microscopy, culture, PCR</td>
<td>frozen</td>
<td>Microscopy - unknown, Culture and Microscopy - 2/3 sediment, In-house PCR - 1/6 sediment, Gen-Probe AMTD - 1/6 sediment</td>
<td>Commercial Gen-Probe Amplified MTD test</td>
<td>39a 32b</td>
<td>95a 97b</td>
<td></td>
</tr>
</tbody>
</table>

* Culture reference standard
* Clinical reference standard
microbiological investigations. In one study, sputa were homogenised by vortexing with NALC alone, NALC and glass beads or glass beads alone [102]. Samples were thereafter split in two, serially diluted and cultured onto Middlebrook 7H11 agar [102]. The authors concluded that chemical and/or mechanical processing of sputa is equally effective at recovering viable tubercle bacilli and that there was no significant difference between the three methods [102].

Another study investigated the use of glass beads with and without DTT for the recovery of bacteria (not M. tuberculosis) from sputum. Glass beads alone were not as efficient for recovery of Haemophilus influenzae as glass beads with DTT treatment (3.8 × 10⁸ CFU/ml vs 5.2 × 10⁸ CFU/ml) [103]. H. influenzae has been previously used as a model for a fragile bacterial cell [104], yet recovery rates were good [103], indicating that fragile cells are able to withstand the harsh conditions of vortexing in the presence of glass beads. In contrast, the cell wall of M. tuberculosis is difficult to lyse (due to its high lipid content) and so likely to withstand this method.

Another method commonly used for mechanical digestion is sonication with the aid of a waterbath. An in-depth description of the mechanism of action of sonication on sputa is described in Baxter et al. Sputa may be sonicated in the presence or absence of a mucolytic agent. Nauwelaers et al. used Ultrasound waves (sonication) to liquefy sputa for extraction of Human Respiratory Syncytial Virus (RSV) RNA. Here DTT and PBS were added to sputa before being sonicated in an Adaptive Focused Acoustics (AFA™) instrument [105].

Although both NALC and DTT are potent mucolytics, they do not digest cystic fibrosis sputum efficiently [99]. However, complete homogenisation was noted when sputa treated with DTT were sonicated intermittently for 120 seconds (30 second intervals) [99]. These fully homogenised samples also showed a reduction in Cq values (mean: 4.25 cycles) by a real-time PCR assay targeting Aspergillus species [99], indicating that mechanical lysis coupled with chemical lysis may improve the sensitivity of NAA tests.

The effect of filtration on sputa

Stepwise filtration of DTT-liquefied sputa with 40, 20 and 11 μm nylon net filters showed a reduction in squamous cells (present in saliva, 30-60 μm in size) but did not affect counts of cells of bronchial origin (approximately 10 μm in size) or differential cell counts [72]. Such filters are therefore likely to allow microorganisms to filter through, provided the microbial cells are not adherent to large cell debris. Filtration may aid microbiological tests by releasing microorganisms from sputa for subsequent culture, microscopy and NAA testing. A filtration system is also integrated into GeneXpert® cartridges. This filter separates the M. tuberculosis bacilli from the rest of the liquefied sputum and its components thereby concentrating the bacilli [104] and eliminating the need for centrifugation.

The impact of sputum liquefaction and extraction protocols on the performance of NAA tests

The methods used to liquefy sputum, extract and purify DNA are likely to have a significant impact on downstream NAA testing. However, variations in study population, sample preparation, sample splitting, DNA target and PCR protocols make it very difficult to compare results on the utility of different methods used for sputum liquefaction and DNA extraction.

Although NALC is generally used for sputum processing prior to NAA tests, DTT has also been shown to be “PCR friendly”. Higher concentrations of NALC (> 0.5 g/L) and DTT (0.1 g/L) have been shown to result in PCR inhibition [107]. Two studies utilising DTT prior to NAA testing for M. tuberculosis, targeting the SecA gene and IS6110 element, yielded sensitivities greater than 95% with culture as a reference standard [108,109]. However, when NALC was used in a multi-site study involving identically spiked sputum, saliva and water specimens, participating laboratories yielded inconsistent positive results (ranging between 2% and 90%) for 103 Mycobacterium bovis BCG organisms [110]. Each laboratory employed their own sample preparation, DNA extraction, and PCR techniques but made use of the insertion sequence, IS6110, as the PCR target, upon request of the investigators. Table 2 provides a summary of four studies that utilised NALC and/or DTT as a mucolytic prior to nucleic acid amplification testing.

Xiang et al. noted a significant reduction in RNA concentration when using 0.1% DTT for sputum processing as compared to specimens not treated with DTT, indicating that even low concentrations of DTT may affect the extraction of RNA [114]. Desjardin et al. showed that NALC/NaOH affects the recovery of M. tuberculosis mRNA but not rRNA. DNA yield was unaffected by the NALC/NaOH treatment [115]. The use of RNA as a target is attractive as there may be multiple copy numbers and RNA can be used to differentiate between dead and live organisms. This may be useful for identifying response to therapy and in patients with a recent history of TB treatment.

Paramagnetic particles (PMP) technology is another means of enriching M. tuberculosis cells and / or M. tuberculosis DNA from processed sputum specimens. PMP’s can be used to concentrate cells and / or DNA instead of centrifugation. TB-beads (Microsens Medtech Ltd, London, UK) used to concentrate M. tuberculosis bacilli from NALC-NaOH processed sputa for smear-microscopy proved to be slightly inferior to conventional concentration by centrifugation (89.4% vs. 91.8% sensitivity) [116], but significantly improved the sensitivity of smear microscopy (P=0.002 and P<0.001) [117,118] when compared with microscopy of unconcentrated sputum. Ghodbane and Drancourt used the same TB-beads for concentration prior to culture and reported comparable results to centrifugation. There are no published results on the use of TB-beads prior to NAA testing [119].
Sputum processing at the point of care (POC)

Resource-poor countries require POC molecular diagnostics that are highly sensitive and specific (thus eliminating the need for culture), inexpensive, robust and simple to perform. These diagnostic tests should be performed at or close to the site of sputum collection and results should be given on the same day.

Mucolytic agents used at POC should be stable as well as safe. In addition, the mucolytic should be able to withstand extreme temperatures. The Xpert MTB/RIF assay reagents are stable between 2°C and 28°C hence its application as a POC test in countries with extreme temperatures is limited to those settings where reagents can be maintained at controlled temperatures.

Another important issue that needs to be taken into consideration for POC testing is biosafety and the use of hazardous chemicals. Patients as well as personnel performing diagnostic tests need to be protected from potentially infectious specimens and harmful reagents or chemicals that might be included in the POC test. Biosafety cabinets are impractical for use in low-income countries as they are costly and require regular maintenance. An example of appropriate processing reagents is the Xpert MTB/RIF sample reagent buffer which is safe to handle (although high concentration of isopropanol requires dedicated shipping due to the flammable risk) and demonstrates 8 log killing efficiency. Sample preparation is quick and easy and can be performed without the use of a centrifuge.

Many current sputum processing methods for diagnosing TB involve the use of a centrifuge to concentrate *M. tuberculosis* bacilli. Resource-poor settings require alternative means of concentrating *M. tuberculosis* bacilli for true POC testing. Alternative methods may include sedimentation, filtration or paramagnetic particle technology, which uses simple magnets to capture *M. tuberculosis* from liquefied sputa.

CONCLUSION

The sensitivity of sputum-based diagnostic tests for *M. tuberculosis* is largely dependent on the efficiency of sputum processing protocols. Efficient processing of samples leads to release of bacteria trapped within the complex sputum matrix. Efficient mucolytics include NALC and DTT. Chemical and/or mechanical digestion can be used; however a combination of both is more likely to result in enhanced homogenisation of sputum. Concentration of *M. tuberculosis* from sputum specimens for subsequent culture or NAA testing can be achieved by centrifugation, filtration or paramagnetic particles.

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