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Sputum Processing Prior to *Mycobacterium tuberculosis* Detection by Culture or Nucleic Acid Amplification Testing: A Narrative Review

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Review Article

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ABSTRACT

Sputum is a complex specimen consisting of a primary network comprised of linked mucin molecules and a secondary network composed of filamentous actin, cell debris and DNA. Other components of sputum are leukocytes, proteoglycans, inflammatory mediators and elastin fibres. Embedded within this matrix are bacteria which are targeted for clinical diagnosis. This is further complicated in tuberculosis as mycobacteria frequently clump within the specimen resulting in unequal distribution. Efficient release of bacteria from sputum specimens requires chemical and/or mechanical breakdown of both primary and secondary sputum networks. This review gives an overview of the composition of sputum and of various methods that have been used for digesting sputum prior to testing for tuberculosis.

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 [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* [3]. However, *M. tuberculosis* is a slow growing organism [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 [4,5] and until recently (with the introduction of the Xpert MTB/RIF test (Cepheid) [6] have not been widely adopted [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 [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 [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 [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 [4,5] however, in practice they generally have suboptimal sensitivity in smear negative sputum specimens [10], 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 [11].

Respiratory mucus forms part of the innate immune system [12]. It is divided into two phases viz, a sol (fluid) and a gel phase [13]. Respiratory mucus is made up of water, ions, proteins, lipids, enzymes, immunoglobulins and large glycoproteins of varying sizes [13-16]. These glycoproteins are called mucins.

Mucins form a dense, protective barrier preventing bacterial attachment to cells lining the respiratory tract [17]. Mucus traps bacteria and debris which are naturally moved out of the respiratory tract by ciliary clearance [12].

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) [18]. The gel forming mucins, MUC5AC and MUC5B, comprise 79% of normal human respiratory secretions [19,20]. The MUC5AC gene is expressed in and limited to the goblet cells of the lung while MUC5B is expressed in the bronchiolar epithelium and submucosal glands [21].

In order for efficient ciliary clearance to occur, mucus must possess high elastic recoil together with low viscosity [22]. 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 [12].

Physiological or structural damage of cilia may occur during lung disease [23] and bacterial infections [24]. When cilia are damaged, ciliary clearance does not occur naturally and the mucus becomes trapped in the airways. In these cases cough may assist with clearance of mucus [23,25,26]. Further, during respiratory disease there may be an increase in the number of goblet cells which results in hypersecretion of mucus [27-29]. Mucin release can be regulated by irritant gases, inflammatory mediators, arachidonic acid metabolites, platelet activating factor, tumor necrosis factor, bacterial proteases, reactive oxygen species, nucleotides, neuronal control and mechanical strain (For review see Kim et. al.) [30]. Cigarette smoke also induces mucus hypersecretion [31]. Mucus builds up in the airways [27] forming a mucus plug [17]. Expulsion of this plug may require expectoration [18]. This expectorate is called sputum [18].

Sputum is a viscoelastic solid [32] made up of mucus [33], leukocytes, cellular debris, bacteria, filamentous actin (F-actin), proteoglycans, DNA, inflammatory mediators [16,17,34] and elastin fibres [35]. Each sputum specimen is unique hence the components present in one specimen may not be a representative of all sputum specimens. The type and extent of a disease (for example necrotising lung diseases such as tuberculosis or pneumonia) may affect the quantity of sputum constituents, such as elastin [36].

The length of actin filaments found in sputum has been shown to correlate with sputum cohesiveness [37,38] although filamentous-actin (F-actin) may not be present in all sputa [39]. Other fibres may be derived from DNA and DNP (deoxyribonucleoprotein) which are released during leukocyte degeneration, to form fibres which contribute to sputum viscosity in purulent specimens [40,41]. These fibres are not present in non-purulent (mucoid) sputa.

Stagnant sputum present in the airways is conducive to bacterial multiplication [42] which in turn attracts leukocytes. The enzyme myeloperoxidase (MPO) is released from degenerating granulocytes in inflammatory states [41,43-45], and imparts a green colour to sputum [46]. Sputum colour is indicative of the degree of bacterial burden [47]. Purulence (green sputum) appears to be associated with increased bacterial load [47-50] whereas mucoid (white, cream) and clear sputum contains fewer bacteria [47,51]. However, this may not always be the case as Brusse-Keizer et al [52]. showed no significant association between sputum colour and bacterial load. In sputum microscopy more than 25 leukocytes per high power field is indicative of infection [53]. In patients undergoing therapy there may be reduced inflammation [54]; improved mucus clearance [54,55]; a decrease in purulence due to a reduced bacterial burden [45] as well as conversion from purulent to mucoid sputum [41].

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 [56]. 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 [57]. 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]. Primestore has some mycobactericidal 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.

Type	Mucolytic	Possible mechanism of action	Result	Ref.
Classical	NALC	Severs disulphide bonds	Reduces sputum viscosity	[78]
	DTT	Severs disulphide bonds	Reduces sputum elasticity more than 90%	[32]
	Thioredoxin	Severs disulphide bonds	Reduces sputum viscoelasticity	[79]
Peptide	DNase	Digests deoxyribonucleic acid/protein fibres	Reduction in viscosity and elasticity	[40, 80, 81]
	Gelsolin	Digests F-actin	Reduces sputum elasticity, viscosity and cohesivity; Drastic reduction in elasticity (77.3%) and viscosity (80.4%)	[32, 37, 69]
	Anionic (Poly) amino acids	Dissolves histones which form DNA and F-actin bundles in sputa, Enhances the activity of Dnase 1	Decrease in sputum viscosity	[39]
	Thymosin b4	Digests F-actin	Decrease in cohesivity, elasticity and viscosity Improves mucociliary clearance in vitro in combination with Dnase 1	[37, 38]
	UFH	Digests DNA and F-actin bundles, Enhances the activity of Dnase	Decreases elasticity but not viscosity	[71]
	Chymotrypsin, trypsin, pancreatin	Breaks down cellular debris	Reduction in sputum viscosity and improvement in expectoration in vivo	[82, 83]
	NaOCl	Breaks down cellular debris	Reduction in cellular debris on smears on microscope slides compared to direct smears	[84, 85]
	USP	May aid in cell lysis, disrupts cell membranes, may reduce cysteine residues	Minimal backgrounds on slides prepared compared to direct smears	[64, 72]
Unknown classification	Modified Jungmann's method	Breaks down cellular debris	Produces a ZN stain "free from artefacts"	[86]
	Chitin	Unknown		[87, 88]
	-with hexa-fluoroisopropanol		Digests sputum more rapidly than NALC in 2% NaOH and NaOCl	
	-with sulphuric acid		Digests sputum more rapidly than 4% NaOH	
	CPC	Unknown	Digestant, decontaminant and preservative for up to 20 days at room temperature	[63, 89-92]
	Iodated compounds	Changes the "potential protein substrate in sputum"	Induces enzymatic proteolysis	[93]
Commercial	Xpert MTB/RIF sample reagent	Unknown	Effectively digests and decontaminates sputum	[94]

CPC – cetylpyridinium chloride; DNA – deoxyribonucleic acid; DNase – deoxyrubonuclease; 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

Classical mucolytics

Classical mucolytic agents, NALC, DTT and Thioredoxin (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 [57]. 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 [59].

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) [75]. DTT has the ability to effectively liquefy sputum, showing a >90% reduction in sputum elasticity [32]. Another study which compared sputum processing prior to cytomorphological 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 sputa 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 dithiols (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.**

DNase 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 digestant 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

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Table 2. A summary of sputum processing methods, and the resultant sensitivities and specificities of PCR methods employed in each of the studies.

Mucolytic		Final Conc. (%)	Ratio	Mechanical	Other	Microbial investigation	Sample fresh/frozen	Sample splitting	In-house/ commercial PCR	Target/ test	Sensitivity	Specificity	Ref.
1	DTT	0.05	1:1	Sonication	boiling	Microscopy, culture, PCR	fresh	Microscopy - unknown, Culture - 200 µl, In-house PCR - sediment (1000 µl), Amplicor - 100 µl	In-house	IS6110	90.76	97.6	[111]
2	DTT	0.05	1:1	unknown	According to manufacturer's instructions		fresh		Commercial	Amplicor	76.92	98.56	
	DTT	unknown	unknown	Sonication	Siliconised glass beads, proteinase K	Microscopy, culture, PCR, gas chromatography-mass spectometry	Fresh. However, samples prepared for PCR were frozen subsequent to processing	Microscopy - negligible, culture - 0.5 ml, PCR - 0.5 ml, gas chromatography-mass spectometry - 1 ml	In-house	IS6110	95	93	[109]
3	DTT	5	unknown	Sonication	SDS-Tris-HCl, zirconia/ silica beads, Nuclisens lysis buffer	Microscopy, culture, PCR	frozen	Microscopy - unknown, Culture - unknown, In-house PCR - unknown	In-house	SecA1	97	97	[108]
	NALC-NaOH	0.5-1	1:1	Sonication	SDS-Tris-HCl, zirconia/ silica beads, Nuclisens lysis buffer		frozen		In-house	SecA1	100	76	
4	NALC-NaOH	unknown-2	1:1	unknown	According to manufacturer's instructions	Microscopy, culture, PCR	frozen following decontamination, microscopy and culture	Microscopy - unknown, Culture - 500 µl, BD ProbeTec - 250 µl, Amplicor - 100 µl	Commercial	Amplicor	89.5	87.5a 100b	[112]
	NALC-NaOH	unknown-2	1:1	FastPrep	lysolyser - heat @ 105°C				Commercial	BD ProbeTec	94.7	86.3a 99.8b	
5	NALC-NaOH	0.5-1	1:1	Sonication	NALC-SDS-Tris-HCl, zirconia/ silica beads, Nuclisens lysis buffer	Microscopy, culture, PCR	frozen	Culture and Microscopy - 2/3 sediment, In-house PCR - 1/6 sediment, Gen-Probe AMTD - 1/6 sediment	In-house	SecA1	24a 20b	95a 99b	[113]
	NALC-NaOH	0.5-1	1:1	unknown	According to manufacturer's instructions		frozen		Commercial	Gen-Probe Amplified MTD test	39a 32b	95a 97b	

[a] Culture reference standard
[b] 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 Haemophilius influenzae as glass beads with DTT treatment (3.8 Í 10^8 CFU/ml vs 5.2 Í 10^8 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 Ultrasone 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 [73]. 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 [106] 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 [94]. 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 [87,120,121], filtration or paramagnetic particle technology, which uses simple magnets to capture *M. tuberculosis* from liquefied sputa [116].

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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