(REVIEW ARTICLE)

Check for updates

Evolution of nucleic acid extraction methods and its role in development of complex human diagnostic solutions with special reference to tuberculosis: A review

Krishna Himmatbhai Goyani [1, 2] and Pratap Narayan Mukhopadhyaya [1, 2, *]

[1] Department of Infectious Diseases, TB Research Section, Wobble Base Bioresearch Private limited, Pune, India.
[2] Institute of Science, Department of Biosciences (Biotechnology), Shri Jagdishprasad Jhabarmal Tibrewala University, Jhunjhunu, Rajasthan, India.

Abstract

Nucleic acid extraction from biological sources has evolved with time and is currently one of the most important contributors to the development of modern human molecular diagnostics. Pure and sufficient quantity of DNA and RNA from limited quantity of cells, which may be fresh or archived, holds key to successful detection of source and cause of many diseases. The challenges of cell disruption to release nucleic acids and the wide range of strategies applied to purify them by removing various enzyme inhibitors are discussed. The demographic spread of the disease of tuberculosis, hurdles faced during transportation of the clinical samples for nucleic acid extraction of *Mycobacterium tuberculosis* pathogen and different detection technologies supported by the extracted pathogen nucleic acid are reviewed.

Keywords: DNA; Purification; Extraction; Detection; Tuberculosis.

1. Introduction

In all form of biomedical research and modern genetic testing, isolation of nucleic acid from clinical samples is an essential and a baseline activity that precede all others. This is considered as one of the most important steps that decide the success or failure of all other molecular techniques [1, 2, 3]. Although the protocols may vary to a significant extent, the success of this process is decided by some key steps which include appropriate and adequate lysis of the biological cells that harbor the target analyte, proper disruption of the nucleoprotein complexes, inactivation of various nucleases that destroy DNA and RNA and an efficient purification process that removes interfering agents that can negatively influence downstream applications [4, 5].

In the broader terms, the isolation of all the three critical biomolecules, *viz.*, DNA, RNA and protein are crucial for modern biotechnology [6]. For a large array of products developed across the world with special reference to diagnostic kits, these steps are primary and important. The target sources for these molecules are varied and ranges from preserved tissues, various kind of cells, viruses and other clinical or biological samples that are required for different analytical or preparative purposes [6].

In the domain of research, two broad categories of extraction processes are encountered, one being extraction of recombinant materials that include plasmids and bacteriophages while the other, genomic DNA of various cells, both prokaryotic and eukaryotic [7]. Further complexities in extraction of nucleic acids commence when the purification needs extend to harvesting of analytes free of proteins, carbohydrates, various lipids and also from other nucleic acids such as DNA free of RNA or RNA free of DNA [8].

* Corresponding author: Pratap N. Mukhopadhyaya. Email: pnm@wobblebase.in

Another aspect that is of immense significance in the world of extraction of biomolecules is its integrity. A high degree of yield of a DNA or RNA without proper structural integrity is not of much use in biological research of clinical diagnostics [9]. RNA, for example, is an extremely fragile and biologically unstable molecule. It remains intact for a very short period of time when extracted from its source (very short half-life) [10]. In biological cells, a wide varieties and types of RNA are present. These include the ribosomal RNA that comprises of around 80% to 90% of the total RNA content. This is followed by the messenger RNA or the mRNA (2.5% to 5%) and the transfer or t-RNA [8]. The methods and processes for extraction and isolation of RNA from biological sources present special challenges due to the omnipresent nature of the RNA degrading enzyme RNAase which is available in virtually all biological samples including bacteria and fungi in the environment [8, 10]. Extraction of this biomolecule therefore call for use of very strong denaturants that kept the integrity of the RNA molecules intact on one hand and prevent the activities of the ubiquitous RNAase enzyme on the other [7]. Added to this, the extraction of RNA always require excellent laboratory practices also.

2. Nucleic acid extraction techniques: Its role and evolution

Although nucleic acid extraction processes are known to the scientific community for decades, there has been a sustained *albeit* slow evolution that has ultimately led to enhanced superiority of the techniques. Research and academic progress in biological sciences has now revealed that nucleic acids or ''NAs'' that exist inside the cell (intracellular) can be grouped into genomic DNA, extrachromosomal DNA and various types of RNA [11]. Despite the fact that RNA and DNA differ in only one type of base where the former contain uracil and the later, thiamine, [12] the basic chemical behaviors of both these NAs are similar. This distinct advantage played a role in the evolving nucleic acid purification techniques and methods and benefitted extraction and purification of both the group of molecules, *viz.*, DNA and RNA.

Popular methods of DNA purification involve electrophoresis, precipitation of the DNA using high salt, the use of spin columns and silica-coated magnetic beads [13]. A large number of commercial companies dominate this space and use these techniques and methods for development of good quality NA extraction kits for use in a NA extraction from a wide range of biological specimen types.

3. Need for indigenization of NA-Extraction solutions in the country

India ranks among the sixth largest country of the world in terms of size. It has a population of around 1.21 billion (http://www.censusindia.gov.in) and is the country with second largest population in the world and home of 17.5% of all humans (http://www.prb.org).

In recent times, data show a decreasing trend in infant mortality rate in India among infants. In this scenario, the role of genetic disorders in deciding the shape of health in the country, is becoming increasing evident and high. This has therefore paved way for large scale genetic testing facility in nook and corner of the country [14].

In all cases, the need for an indigenous nucleic acid extraction tool with scope for continuous improvement and its manufacturing within the country, has never been felt so much before, especially in the backdrop of the recent COVID-19 pandemic in the country [15].

4. DNA and RNA as important components in molecular biology research

In the field of molecular biology, extraction of biomolecules such as DNA, RNA and protein play a very crucial role. This is the starting point for all forms of product development that involve extraction kits for DNA, RNA and proteins.

DNA extraction broadly involve isolation and purification of two forms of cellular DNA, one being plasmids or extra chromosomal DNA and the other, the genomic DNA [7].

Successful extraction of NA from biological cells requires release of the NA from cells with high efficiency followed by removal of contaminants such as proteins and other undesired biomolecules which include carbohydrate, lipids, or other nucleic acids in order to acquire pure DNA without RNA contamination or RNA without DNA contamination.

DNA is a stable molecule and can withstand harsh extraction conditions. On the other hand, RNA is relatively unstable and with much lesser half life time period when out of the cellular environment. Therefore, extraction of RNA requires additional care and caution compared to its DNA counterpart. An important and challenging aspect of RNA extraction is the presence of the RNA degrading enzyme that is ubiquitous in nature, heat resistant and known to refold after denaturation. Hence, kits involving extraction of RNA require specially treated water, reagents and labware.

4.1. History of DNA extraction and current trends

DNA extraction from biological samples was first attempted by a Swiss Physician named Friedrich Miescher in the year 1869 with an aim to determine the chemical composition of human cells. Since then, the process of nucleic acid extraction has undergone sea change with new discoveries and inventions adding convenience, efficiency and economy to the already existing protocols.

4.2. Evolution of nucleic acid extraction process and techniques

Since the first discovery of extraction of DNA from clinical samples, the initial regular process of DNA extraction and purification was by density gradient methods. Later, the differential biophysical behavior of extrachromosomal DNA and chromosomes at alkaline environment was exploited for the isolation and purification of different types of DNA.

4.3. The modern methods of DNA and RNA extraction

The current methods of DNA and RNA extraction broadly hover between a few different technology platforms. These include solution-based methods, use of various silica coated natural and artificial solid supports which later paved the foundation for solid phase purification method, spin-column based procedures and silica-coated magnetic bead-based technology.

4.4. The Solution based method

This method involves the use of Guanidinium Thiocyanate-Phenol-Chloroform Extraction process. It is now widely known that salts are one of the most common impurities in NA extracts and hence there is a need to remove them prior to its use for any downstream application. This call for a single or more than one processes or steps for purification where the DNA is desalted prior to final use [16]. The other steps of NA acid extraction, *viz.*, cell disruption, inactivation of cellular nucleases and separation of desired nucleic acid from the debris remains more or less the same.

4.5. Phenol Chloroform as a medium for DNA and RNA purification

In the solution method, purification of DNA and RNA using phenol chloroform is one of the most popular steps followed by the scientific community. Phenol is an inflammable, corrosive and toxic acid (carboxylic acid) and is known to denature proteins completely. However, an important shortcoming of this chemical is that it only partially inhibits RNAse activity that is crucial for RNA extraction [17]. This problem eventually was resolved by using a mixture of phenol, chloroform and isoamyl alcohol at a ratio of 24:24:1. Using this combination, all contaminants that mainly comprise of proteins, carbohydrates and degraded cell wall materials after cellular disruption remain in the aqueous phase after lysis of the cells. However, after addition of phenol, chloroform and isoamyl alcohol mixture, these contaminants migrate to the hydrophobic phenol portion that is heavier and hence at the bottom of the tube while DNA remain in the hydrophilic aqueous upper phase. This upper phase is then collected and DNA is precipitated in presence of a salt and ethanol. This method of DNA and RNA extraction still remain the method of choice in many research laboratories working in the field of molecular biology.

4.6. RNA extraction process evolved to address the intrinsic problem of RNA-instability

The extraction of RNA from biological samples requires the use of a specific chaotropic agent represented by salts of Guanidine. This method was first described by Ulrich and co- workers (1977) [18]. However, the steps involved in use of this chemical [Guanidine thiocyanate] along with phenol chloroform was found to be time consuming and laborious. This was eventually replaced by use of a single reagent that comprised of guanidine thiocyanate and phenol as a mixture and was developed by Chomczynski and Sacchi (1987) [19]. In this process, the lysed biological specimen was mixed with the solution at an acidic pH environment. Guanidine salts are chaotropic in nature and hence promote protein denaturation.

This method of RNA extraction resulted in physical separation of RNA after lysis in aqueous environment in an acidic condition and is widely used as a method of RNA extraction worldwide [17]. This is followed by recovery of the RNA molecules by adding isopropanol at 1:1 volume and 1/10 volume of sodium acetate or equivalent salt.

4.7. Alkaline lysis: The method to extract small extrachromosomal DNA

With the advent of recombinant DNA technology, the need for extracting pure plasmid DNA became a priority. In order to address this, the alkaline method of plasmid DNA extraction evolved. This relied on the fact that covalent circular plasmid molecules denature at high pH (>13) and renature immediately when neutralized [20]. On the other hand, the

large, sheared genomic DNA molecules denature and remain in that state and eventually form complex with sodium dodecyl sulfate and precipitate [17].

4.8. Ethidium Bromide [ETBr] -Cesium Chloride [CsCl] Gradient Centrifugation is a potent method of DNA and RNA purification

Close to the alkaline method of plasmid DNA extraction and purification, yet another effective *albeit* expensive and tedious method of plasmid DNA purification evolved in the map of molecular biology techniques [21]. This method relies on the fact that DNA after incorporation of Ethidium bromide acquire a specific and precise molecular weight that dictate its alignment in a specific position in the Cesium chloride gradient slant generated by controlled centrifugation. This allows DNA molecules of varying sizes to physically separate, where each layer at a specific location in the Cesium chloride gradient thus allowing their isolation & rescue by puncturing the plastic tube with a sharp needle attached to a syringe.

4.9. CTAB method evolved as a prominent DNA extraction protocol

When plant molecular biology research took pace, there was a need to extract high quality plant DNA and RNA. However, it was eventually realized that plants contain high quantity of polysaccharides. This led to the use of the chemical called Cetyltrimethylammonium bromide (CTAB). This is a non-ionic detergent that precipitates DNA & RNA and other acidic polysaccharides from low ionic strength solutions. However, at high ionic strength solution, CTAB fails to precipitate the nucleic acids and instead forms complex with proteins. This method is therefore very useful in extraction of DNA and RNA from plants and certain specific types of bacteria where large quantity of polysaccharides are released during cell lysis [22].

4.10. The emergence of Solid-phase Nucleic Acid Extraction

With time, the inconvenience in handling solution-based DNA and RNA extraction and purification methods, was acutely felt. This paved way for an entirely new concept of DNA purification method that exploits a solid phase for the process. In fact, most of the modern-day DNA and RNA extraction kits currently use this technology. The method is rapid, convenient and efficient. In this process, the solid phase system absorbs the nucleic acid as a result of hydrogen – binding interaction with a matrix that is highly hydrophilic under chaotropic salt conditions allowing all other contaminants to get washed away except the nucleic acid molecules. The DNA or RNA is thereafter eluted from the silica solid support using a low salt buffer [23, 24].

4.11. Different types of silica- coated solid support formats are in vogue

A large number of different silica -coated DNA & RNA purification modules are used in modern times. Some prominent formats among them are as follows:

4.11.1. Silica matrices

These are prepared by refluxing silicon dioxide in sodium hydroxide or potassium hydroxide at a molar ratio of about 2:1 to 10:1 for 2 days. In this method, the DNA binds to the inorganic matrix and is thereafter released when treated with hot water [25].

4.11.2. Glass particles

In this method, DNA released from agarose gel, for example, are made to adhere to glass particles, powder or glass beads along with silica gel. While the principle for adhering of DNA remains the same, this is still a popular method of extraction and purification of DNA from biological sources [26].

4.11.3. Diatomaceous Earth

Diatomaceous earth, which is also known as kieselguhr or diatomite is known to have a very high silica content of around 94%. This therefore functions as an excellent solid support for extraction of DNA and RNA. The soil is mixed with crude cellular lysate containing the DNA or RNA under high chaotropic salt condition, which allows binding of the DNA/RNA to these silica-containing earth powder. Following rigorous washing, the bound nucleic acid is eluted using a low salt buffer [11].

4.11.4. Magnetic Bead Based Nucleic Acid Purification

The solid phase DNA and RNA purification technique underwent a sea change with the growth of knowledge of silica's DNA and RNA binding capability under specific chemical conditions. However, this technology reached its peak with the

knowledge that small magnetic beads can be coated with silica [27, 28]. This method allows temporary capturing of the DNA and RNA-bound beads to the wall of the tube by a magnet thereby giving the opportunity to wash away all residual impurities that remain in the solution. This can be repeated, theoretically, many times, to acquire desired purity of the nucleic acids. The captured molecules can then be released by detaching the magnet and adding a low ionic elution buffer. This releases the captured DNA into the added buffer. The nucleic acid-free iron particles are then captured again using a magnet and the DNA-containing buffer is removed to store or proceed with downstream applications.

4.11.5. Spin columns

This technology involves use of cellulose membranes coated with silica or glass fibers that are loaded on to spin columns. DNA containing lysed cell suspension is passed through these columns using centrifugal force and in the presence of high concentration of chaotropic salts. The DNA binds to these silica-coated membranes while the contaminants are removed by repeated washing using ethanol-based wash buffers. The DNA thereafter is released by passing through a low ionic buffer such as water. This is one of the most popular methods of DNA and RNA extraction in current times [29].

5. Biological cell wall disruption

Breaking of cell or its disruption involves destruction of the outermost layer of the cell that allow release of nucleic acid present inside it which is then amenable to purification and isolation. In the field of molecular diagnostics, breaking of cell wall play a crucial role and is often the first most important step towards a successful diagnostic experiment.

In the domain of biological experimentation, cell lysis may be either partial or complete in nature. Partial cell lysis, involving partial rupturing of cells using thin glass pipettes, is performed in procedures that involve 'patch clamping' and is a method of choice in assays related to testing of drugs and determination of strength of ions in a solution [30].

However, from this study's perspective, complete cell lysis is more important since it allows release of the entire nucleic acid content inside the cell into the suspension medium [31]. Given the fact that a range of micro and macromolecules in the form of DNA, RNA and several sub cellular components are simultaneously released, in molecular diagnostics, complete lysis of cell is almost always followed by purification of the target analyte prior to testing [32].

The international market shows strong trend towards technologies that are related to extraction of nucleic acid after its lysis with a projected business volume of 2.36 billion dollars in the year 2016 and 3.85 billion dollars in the year 2021 [33].

In recent times, the process of cell lysis followed by purification of the target nucleic acid analyte has undergone dramatic reduction in volume of samples also, that are handled. The concept of 'nano' or 'micro' has made inroads in this technology domain in a big way giving birth to an entirely new dimension of cell lysis and downstream applications at miniature scale which is now known as the 'microfluidics' [34].

6. Role of human DNA in addressing critical diseases

A wide array of genes are now known where mutations are unequivocally linked to higher risk of cancer [35]. Despite the fact that a detailed pedigree analysis followed by conducting a genetic test is (often) expensive when compared to conventional therapy, it is often justified due to some obvious advantages associated with it [36].

The key to a good genetic report lies in the availability of good quality DNA or RNA as the case may be. Importantly, almost all techniques that utilize DNA as an analyte to detect or diagnose a disease rely on the uniqueness of the nucleotide sequence within a strand of DNA. Therefore, an array of detection technologies evolved once extraction of pure DNA from human cells was optimized.

One of the first technology to capture this featured was called the DNA-SSCP (single strand conformational polymorphism) where alteration of a base of the mutated gene fragment compared to its homologous counterpart resulted in reduced annealing temperature that made them assume a different conformation that was eventually captured by gel electrophoresis. [37]. The other methods similar to this are the HET or the heteroduplex analyses [38], chemical mismatch cleavage [39], the method of denaturing high-performance liquid chromatography [40] and the DGGE or the denaturing gradient gel electrophoresis [41].

Denaturing high performance liquid chromatography or the DHPLC is an useful DNA screening system for interrogating initial alterations in DNA that can result in aberrant functioning of a gene [40, 42, 43, 44, 45]. The principles that govern DHPLC is similar to that of heteroduplex analyses but involve highly specialized columns that has the capacity to separate DNA which differ from its counterpart by as little as a single base. In this technique amplified segment of DNA obtained from pure clinical DNA is denatured in a gradient environment. It has a solid phase which has higher affinity towards perfectly renatured DNA compared to heteroduplexes. The separation of heteroduplexes is monitored using the 260 nanometers absorption property of DNA. Data suggest that this is an efficient and reproducible method for screening mutations within a specific DNA fragment [46, 47]. The sensitivity and specificity of this method has been reported to be high and reliable [40, 44, 45].

With the advent of DNA sequencing, this technology has gradually assumed the position of 'Gold standard' in the field of genetic testing. Since the inception of this technology in the domain of genetic testing in the year 1990, there has been a steady and gradual progress with special reference to instrumentation and chemistry. This led to the development of automated genetic analyzers that not only increased the speed but also the accuracy of DNA testing in the world of molecular diagnostics [48].

In recent times, the DNA sequencing capabilities has dramatically improved with the advent of the next generation sequencing platforms. This has potential to reduce DNA sequencing cost in near future. Several platforms has evolved with time in this domain such as the Roche 454 and GS Junior system [www.454.com], the Illumina Genome Analysers-HiSeq and MiSeq [www.illumina.com], Ion Proton and Ion torrent [www.iontorrent.com].

Although the general perception about next generation sequencing [NGS] platforms is about sequencing of the whole genomes, several applications are available now that allow experiments to be performed that are similar to traditional DNA sequencing such as resequencing of genes for a targeted mutation [49].

One of the significant advantages of the NGS platforms is its ability to extract information from human DNA by analyzing selected panels of genes both of somatic and germline origins where all targets are processes in parallel [50, 51, 52]. This is particularly relevant in cases of cancer where a large number of genes collectively dictate disease cause and progression. With ongoing developments in the domain of NGS, new techniques are available now where the native DNA can be processed without any sort of preamplification by a process what is now known as single molecular sequencing (www.helicosbio.com). Another similar technology is the GridION system that is based on NanoPore technology developed by Oxford NanoPore Technologies, located in Oxford, United Kingdoms and the SMRT TECHNOLOGY (www.pacificbiosciences.com).

One of the greatest contributions of pure extracted human DNA to mankind is its potential to throw light on genetics of oncology. In the last decade and a little more, there has been a conscious departure from the theory of "one size fits all" in addressing oncology treatment to customized and highly individualized therapy. This paved way for the advent of precision medicine where genomic variants are carefully analyzed to decide a therapy. Precision medicine is the process or method of taking therapeutic decisions after taking into consideration the genetic variation or variation of gene expression of a subset of patients who all are expected to benefit the most from the proposed individualized therapy [53].

It is now more than 50 years that circulating cell-free DNA (cfDNA) is contributing to oncology management. These are DNA molecules that originate from healthy as well as diseases cells and are found circulating in the blood stream. There are a number of processes and events by which cell free DNA can be released from cells. These can be active secretion, necrosis or apoptosis. The process of necrosis releases DNA fragments that are larger in size (greater than 10, 000 bp) while the process of apoptosis releases DNA fragments of shorter length and in the range of 180-200 bp [54, 55] that is similar to the length of DNA molecule that is wrapped around one single nucleosome. Longer cfDNA are found but their quantity is lesser in comparison to the average range of cfDNA. Physiological events such as exercise or infection releases more cfDNA into the blood stream. However, the exact process of removal of cfDNA from the blood stream and its elimination is not clear. Not only kidney but organs such as liver and spleen has been discussed for its contribution in this elimination process of cfDNA. However, it is important to note that the average half-life of these cfDNA population is only 90 to 120 minutes [56].

There is another similar category of DNA that has been refined for its extraction process and is immensely contributing in the domain of oncology diagnostics. These are the circulating tumor or the ctDNA These short DNA fragments are released from human tumor cells and are a part of the overall cfDNA population in the blood. These categories of DNA carry vital genetic information embedded within them which include point mutations, rearrangements of the chromosome, variation in copy number and several others. Interestingly, the large proportion of ctDNA does not

originate from the circulating tumor cells. Instead, they originate from cells residing in solid tumors and are released through the mechanism of apoptosis and or necrosis [56]. Two types of patients show elevated cell free DNA. Apart from cancer patients, the other one includes pregnant women with fetal DNA that is also used for important diagnostic purposes [57].

The technical advances seen in modern oncology diagnostics owes a lot to the technology of extraction of various categories of DNA available from human body that has clinical diagnostic relevance [58, 59, 60, 61, 62, 63]. In recent times an alternative to tissue biopsy has come into practice by way of liquid biopsy. This technique has the potential to avoid specific disadvantages related to tissue biopsy such as bias in sampling, heterogeneity and intra-tumoral environment and obstacles pertaining to repeat DNA extraction from clinical samples [64, 65, 66].

The natural properties of cfDNA such as its short shelf life make it an immensely challenging analyte to extract. However, the primary obstacle stems from its low concentration in human blood that averages to a low of around 30 ng/ML and within the range of 1.8 to 44 ng/ML [67, 68]. However, the mean concentration of cell free DNA in patients suffering from cancer is around 180 ng/ML [69, 70, 71, 72]. Nevertheless, this quantity too remains challenging to detect. Adding to this difficulty is the low proportion of circulating tumour cell DNA that is around 0.01% of the total cell free DNA [73, 74, 75, 76]. After a good extraction of this category of DNA, the other major obstacle faced is the intense background of the cell free DNA that obscure the clinically important circulating tumour cell DNA. The short half-life of this category of DNA also makes it imperative that the extraction of DNA is performed fast and quick owing to their short half-life [61, 63, 77, 78]. Any fault in ctDNA extraction and detection can lead to mis diagnosis of cancer. Given the fact that polymerase chain reaction and all other downstream process of detection occurs after the extraction process, a good extraction is imperative for a sound cell free DNA based clinical diagnostics with special reference to oncology. In the present times, cell free DNA is typically extracted using silica-based spin columns, magnetic beads and the classical phenol chloroform based organic methods [63]. Among all these methods, the most popular is the spin column based one that is widely used across the world [79, 80, 81]. In this process, the DNA gets attached to the silica membranes in the presence of high concentration of chaotropic salts. This binding is reversible and can be altered to release the DNA by washing with a low salt buffer [82, 83].

Despite the fact that the spin column-based method of extraction generates high quality cell free DNA, the method has distinct disadvantages. It requires a high-speed centrifuge for processing the clinical sample, uses a range of solvents and buffers that are to be carefully and sequentially added to the tubes, calls for manual handling that brings in the chances of cross contamination and requires a skilled operator to perform the processing steps [84]. To address these lacunae, methods based on microfluidics has come into vogue that use further advanced methods of DNA extraction from biological sources. This includes the use of extraction methods that employ various solid phase microfluidics and employ micro-channels with extended surface areas, small, miniaturized fluid chips [85, 86, 87] and similar other methods mostly involving silica membranes [88, 89] or silica beads [90, 91, 92].

Newer methods of extraction of cell free DNA are being experimented in recent times. This include PIBEX technology (Pressure and immiscibility-based extraction) which is a method devoid of any centrifugation steps [93] and a lab-on-a-disc system [94].

7. Detection of human pathogens using DNA & RNA as an analyte

The inability to take clinical decisions based on detection technologies which were not rapid neither accurate in identifying a pathogen in the past era led to most clinicians follow a very conservative therapeutical management approach. However, the benefit of this approach if any, were balanced by strong negatives such as empirical treatment, medical complications and unnecessary treatment, hospitalizations and emergence of drug resistance [95, 96, 97].

For a long time, molecular techniques such as polymerase chain reaction were projected as tool of the new generation with potential to make the existing classical diagnostic processes vintage and obsolete [98, 99, 102]. As this technology migrated, gradually from bench to bedside, the clinicians acquired greater spectrum of knowledge to decipher and use the data for the benefit of the diseased community [100].

With time, PCR evolved to accommodate larger spectrum of problems and generated wider range of data in a shorter span of time. The multiplex PCR, for example, interrogated multiple targets to generate data from each one of them at one go [101]. Repeat PCR using closely related PCR primer sets resulted in development of the nested PCR protocol that dramatically enhanced sensitivity and specificity [102]. The specificity of a reaction was strategically reduced to capture larger spectrum of genetic information within a wider group of organisms [103]. Detection of pathogens having RNA as their genome was made feasible because of the discovery of the process of reverse transcription that led to the

development of the reverse transcription PCR or RT-PCR which was instrumental in a big way in detecting RNA viruses [102].

However, the most dramatic evolution of the PCR process is the transition from conventional end point PCR to real time PCR technology. Here, both the amplification as well as the detection steps are integrated within a single reaction tube thus doing away with the laborious agarose gel detection method. Thus, it diminished the post amplification processes to near nil, restricting it to mere analysis of data using a wide array of software. Most importantly, real time PCR allowed accurate quantification of the targets [104].

Gradual evolution of technologies for extraction and purification of nucleic acid from human pathogens perfectly complimented the same in the domain of PCR technology with real time PCR emerging as a powerful method to detect such pathogens when pure and sufficient analyte is provided.

Therefore, a careful investigation into economic methods of development of DNA & RNA extraction methods should be duly complemented with development of customized real time PCR technology also to detect clinically important diseases such that economy and technological development occur at the same time and provide collective benefit to the community.

Different anatomical regions of human body are colonized by microbes which play vital role in deciding health conditions as well as diseases of the host [105]. To cite an example, microbes residing in the human intestine aid in processing compounds inaccessible to the human physiological environment [106] and also in maintaining host immune health [107, 108]. Similarly, the microflora of the vagina in human females assist in preventing diseases of the urogenital system and also in maintaining overall health of women [109, 110, 111].

In recent times there has been an increased interest in understanding the difference between individuals or groups of individuals that decide risk of a disease or its progression. In order to achieve this, a sound knowledge of the differences in microbial flora present within each individual, its diversity and distinctness play a crucial role. In the past several decades microbial information that evolved from cultivation-based methods provided invaluable information. However, they are mostly incomplete and fail to reflect the exact diversity prevalent in the flora. Further to this, a vast number and type of microbes still remain that are refractive to culture and cultivation and hence inaccessible to laboratory experimentation-based methods to understand them [112, 113, 114, 115].

This disadvantage led to development of molecular methods that analyze the 16S rRNA gene in order to understand microbial phylogeny and more importantly, this method can be adapted to microbes that cannot be cultured or cultivated in the lab [116, 117]. Therefore, this technique is adopted by a vast majority of laboratories that are interested in studying microbial community residing in the human body [118, 119, 120].

Most if not all of these methods of microbial analysis require DNA to be extracted from the microbial cells. Extensive studies have demonstrated that bacterial cell wall and its structure has a profound effect on the efficacy of extraction of DNA from the cells [121, 122, 123]. This issue can have serious repercussions as it has the capability to distort the representation of a specific portion of the microbial community found in it's natural flora [121, 124, 125, 126, 127, 128] and introduce unintended bias into the composition of the analyte [121, 123, 129]. However, despite this critical aspect, inadequate attention is received by the process of DNA extraction from mixed group of microbes [122, 130] which were eventually used to generate academically important data without proper validation of the composition of the extracted DNA.

There are multiple ways by which quality of extracted DNA can be evaluated. This include assessing the yield of DNA, measuring the shearing quotient of DNA and reproducibility of the extraction process. Physical method for lysing bacterial cells is very effective. However, one of the downsides of this method is intense and over shearing of the genomic DNA within the cells that generate smaller DN fragments. Such small fragments fail to respond to polymerase chain reaction that target a gene by amplifying a reasonably longer stretch of DNA [131, 132]. Having taken all points into consideration, a proper and accurate representation of DNA from all member microbes of a mixed community remains the most important criteria while analyzing microbial flora [124, 133]. This is so because the ultimate aim is to analyze DNA that has proper representation from all microbes in the mixed community without any bias of abundance or composition. However, most of the studies in this domain occurred on environmental samples where the true composition and abundance of any one microbe remains unknown thus making proper evaluation of appropriate representation of all microbe present practically impossible [121, 134, 135].

It is now abundantly evident that with the advent of improved methods of DNA extraction from living cells and evolution of complex and sophisticated DNA analytical methods, the field of medical research has benefitted immensely including the area of human microbial pathology. Techniques such as DNA sequencing, polymerase chain reaction (PCR), techniques related to DNA-DNA hybridization are now used extensively across the world for identification of microbial species. If seen closely, it will be found that a large number of bacterial species has been identified in recent times which are exclusively based on molecular data originating from DNA within them [136, 137].

While a majority of original methods for lysing of bacterial cells relied on organic extraction, additional agents to aid lysis of the bacterial cell also came into use. This include lysozyme, Tween 20, sodium hydroxide, sodium dodecyl sulfate, proteinase K, Triton X 100 and guanidine isothiocyanate [138, 139, 140, 141, 142, 143, 144, 145, 146, 147]. Apart from these agents, physical forces such as irradiation by use of microwaves, ultrasound waves, heating and cooling, use of magnetic fields and use of heat exchanger coils are also used in different proportions [138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151]. There are several protocols available that use all approaches, namely chemical, enzymatic and physical methods of lysing bacterial cells [138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152]. However, from a commercial point of view, most of these protocols are expensive as well as time consuming. To address this issue, several newer methods have evolved, some of them have been automated and are quicker and simpler to process from the operator's perspective. Nevertheless, cost continued to remain high for most of these protocols unless used in large volumes [147, 153].

An area where extracted pathogen DNA has immensely contributed to human health is in the domain of food borne pathogens. Contamination of food with pathogenic bacteria is associated with human mortality, animal suffering as well as significant burden to the country's economy. Most outbreaks have common symptoms that is seen by way of vomiting, fever and diarrhea which makes identification of the exact causal element difficult. The strong economic impact of food borne pathogens indicate the need for public awareness and safety. This is indicated by the food safety market of Europe that is primarily dominated by of food-testing technologies. This market has already reached around $4 billion in the year 2018 and the anticipation for year 2025 is around $6.5 billion [154]. Though the global rate of incidence of food borne diseases is difficult to ascertain, European Food Safety as well as the European CDC (Centre for Disease Prevention and Control) reported around 359, 700 cases of hospitalization and around 500 mortalities in the European Union in the year 2016 [155]. Using extracted DNA from the pathogen or its sources, methods such as use of sensors, nanotechnology and micro fluidics has recently added great value to DNA based diagnostics of food borne human pathogens [156].

Milk has been the home to pathogens that are very harmful to humans. Human population has been recorded to consume milk of several other mammals since a long period of time [157]. The main dairy animals that are recently recognized across the world are cattle, goat, water buffalo and sheep that collectively produce over 850 million tons of milk in a year [158].

Good level of nutritional content in milk makes it an excellent drink for human consumption. However, the downside is that its complex composition with very high fat content and a pH value that is close to neutral also provides an excellent environment for microbial growth and metabolism [159, 160]. Milk has an abundance of lactic acid bacteria that is beneficial to human health on one hand, but also allow growth of harmful pathogens that can spoil milk and cause human diseases [160, 161].

There are a variety of techniques by which bacterial organisms present in raw milk can be detected and identified [162]. These are primarily either phenotypic or genotypic methods. Phenotypic methods, mainly rely on culture-based techniques, are economical and cost effective but requires significant man-hours to perform and also consume a long period of time. Added to this, there is occasional absence of sufficient information generated to discriminate one type of pathogen from the other.

With the advent of improved DNA extraction techniques coupled with newer DNA based analytical methods, various supporting technology platforms has now evolved that can be used for confirming the information generated by traditional phenotypic methods [160, 162, 163]. A large number of DNA based analytical methods has developed over time to genotype pathogen present in milk. Interestingly, most if not all of these techniques rely on polymerase Chain Reaction or PCR that is now acknowledged as the central backbone of present molecular biology field. All of this is possible owing to the availability of good DNA extraction and purification methods that is dominated by silica membrane-based columns which produce excellent quality DNA for such purposes, although they are relatively expensive compared to extraction method by older techniques [164, 165, 166].

From the point of view of PCR, raw milk is notorious for the presence of a large range of chemicals that function as potential inhibitors for the *Taq* Polymerase enzyme-based reactions. Hence in almost all cases, there is a need for incorporating a specific step that is aimed exclusively to remove such inhibiting components or at least inactivating them such that they do not interfere with the process of PCR [167, 168]. This is precisely the reason why methods such as removal of apoproteins and components of the membrane of the fat globules by selective solubilization is performed using a range of nonionic and ionic detergents [169, 170]. One of the most effective such nonionic detergents is Triton X 100 that is used to treat bacteria harvested from raw milk for genetic analysis [171]. Apart from this, use of magnetic bead-based technologies and several other home-brew methods are also available for improving the quality as well as the quantity of extracted DNA [165, 172, 173, 174].

Since the advent of prokaryotic life on the earth almost 3.5 billion years from now, microbes remain the most widely found living organism [175, 176]. Given the fact that DNA is a very stable molecules and has the potential and capacity to remain in nature for thousands of years, one can find traces of such DNA in almost all places on earth's ecosystem. With the development of sensitive molecular biological technique such as 16S ribosomal RNA gene sequencing and the technique of shotgun gene sequencing using automated genetic analyzers, it is now possible to identify the lineage of a DNA also that is found in the nature's ecosystem. Very importantly using these sensitive techniques, it has now been found that reagents and chemicals used for extraction and purification of DNA from biological cells and a host of other reagents used in the process of genetic analysis of DNA, are contaminated with DNA originating from different bacterial sources [177, 178, 179, 180, 181].

There are a large number of research articles and publications that describe potential contamination of PCR and allied reagents with microbial DNA owing to their wide spread presence in various laboratories due to microbial DNA based research underway. Quite expectedly, several methods too have been prescribed from time to time to address this issue such as irradiation of reagents with ultraviolet light, treatment with different restriction endonucleases, treatment with ethidium monoazide [EMA], etc [181, 182, 183]. However, none of the methods proved to be reliable, consistent and durable. Despite this obvious knowledge prevalent in literature very few if any laboratories working on DNA sequencing of microbes use appropriate control to monitor such contamination or take steps to eradicate any possible microbial DNA contamination in the reagents [184]. Needless to say, this contamination is the cause for several reports of microbe-DNA data appearing in literature that surprisingly match with data obtained from apparent negative controls [184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196].

8. Tuberculosis and India

This disease remains in the forefront of Indian health priorities for years together now. In the world, there were an estimated 10.4 million new cases and 1.8 million deaths resulting due to this disease as on year 2015. [197]. Out of this population, 2.8 million new cases have been documented to occur in our country with an estimated 0.48 million deaths per year because of this disease [198].

In the south-east region of Asia, out of the first six countries with very high burden of tuberculosis, India accounts for 28% of it [199]. As per the National TB prevalence survey published in the year 2021, the occurrence of tuberculosis infection in people with age less than 15 years was 31.3%. Further, around 5 to 10% of them developed the active form of the disease [200]. When a person infected with tuberculosis gets delayed diagnosis and treatment, he/she continues to infect newer people with the pathogen thereby creating permanent reservoirs of the microorganism in the society [201]. Therefore, people suffering from tuberculosis are always a threat to the society owing to its potential to maintain a perennial source of infection in the population by the time he or she is diagnosed and treated. This is precisely the reason why, breaking this chain of passing on the infecting capability from one individual in the population to another is one of the main aims of the World Health Organization-supported End TB Strategy [200, 202].

Addressing the challenge of controlling tuberculosis by blocking active tuberculosis pathogen infection is poorly discussed in our country. None the less, it remains one of the crucial components in our National Strategic Plan 2017-2025 to irradicate the disease in India by year 2025 [203]. A report by the lancet Commission on tuberculosis states that all strategies for diagnosis and treatment for this disease are likely to fail unless an appropriate preventive protocol for spread of the disease is adopted in the comprehensive strategy to control tuberculosis. [204].

8.1. Challenges posed by tuberculosis in the country

The increasing rate of ageing population in the country coupled with emergence of drug resistant tuberculosis is a new concern in India [205, 206]. In routine clinical practice, detection of tuberculosis is challenging since it does not display any critical symptoms until late-stage progression of the disease. Nevertheless, a prompt detection when the disease is

at its initial stage, is key to success in controlling tuberculosis in India and elsewhere [207]. In India, a low socioeconomic status and sub optimal conditions of living coupled with poor nutritional food habits are believed to be the most prominent factors enhancing the risk of spread of tuberculosis in the population. [209]. While the government takes disciplined steps to administer the BCG vaccine, its advantages are minimal compared to the spread of the disease and its effect gradually decline as the age of the individual progresses [208]. Added to this, the issue of latent tuberculosis is also emerging as a potential treat in the older population of the country. Although tuberculosis is seen to occur in almost all countries in the world and also, in all the age groups, but 90% of the cases appear to be adults. Among this, almost two third is found in around eight developing countries in the world with India being one and contributing to almost 27% of these cases [210, 211].

Reporting of cases of active tuberculosis is also a potential issue that hinders control of the disease. In the year 2017, only around 64% of all the incidences of tuberculosis in the world were reported and almost 36% of the cases were either not diagnosed, left without any treatments or were not reported at all. These so called "missing cases of tuberculosis" has raised great concern amongst agencies involved in eradication of the disease [212]. Given the fact that tuberculosis is more prevalent in developing countries, a large section of the diseased population does not have proper access to adequate healthcare facilities. As a result, most if not all are reliant on sputum microscopy as the sole mean for detection and diagnosis of tuberculosis. However, it is now widely known that these techniques, although economical and deployable in remote areas, has its own limitations, sensitivity and inability to distinguish different strains of the pathogen and its drug resistant variants being some of them [213]. Another major challenge faced by governments in their effort to irradicate tuberculosis is the unplanned and improper use of anti-tuberculosis drugs. This is one of the primary reasons for development of the multi drug resistant and extensively drug resistant *Mycobacterium tuberculosis* variants in the population. In this context it may be noted that India has one of the highest incidences of multi drug resistant and extensively drug resistant tuberculosis cases in the world when compared to rate of incidence of "regular" tuberculosis. [214, 215]. Needless to say, missing medical appointments for several months while on treatment is one of the most serious issues for individuals as well as the society as a whole in the domain of tuberculosis disease [216]. The emergence of multi drug resistant tuberculosis makes treatment prolonged and difficult. Its rising rate of incidence particularly in the younger population of the country hence, is a major concern and a tall hurdle in the effort to irradiate the disease from the population [217]. The other challenges are prevalence of large section of the tuberculosis patient population in rural and remote areas of the country with poor access to adequate healthcare, available private healthcare sector being largely unorganized, absence of adequate political will to irradicate the disease that is shown by various governments from time to time and prevailing corruption in the society [218]. In this context, the Stop TB campaign of the World Health Organization is a fresh, and welcoming endeavor to eradicate this disease from the earth by year 2050 [218].

Around year 2000, world has seen rapid surge in technologies for diagnosis of tuberculosis. Added to this, new and more effective medicines were developed by way of Delamanid, Bedaquiline and Teixobactin [219]. However, poor access to these newer developments remains the main hurdle in eradication of the disease from the low- and middle-income group of countries in the world, [220].

In India a National Strategic Plan [Year 2017 to Year 2025] has been adopted by the government which is a strong step towards rapidly reducing the rate of tuberculosis incidence in the country. These measures are in line with the globally launched End Tb targets and goals that collectively aims to have a TB Free India. This program aims to first rapidly reduce the incidence rate of the disease followed by its eradication by year 2025 [221]. In the year 2015, India already touched the ambitious target of reducing the tuberculosis rate of incidence by half. However, the role of drug resistant tuberculosis emerged as a major challenge in this path to achieve a TB Free status in the country.

8.2. Transportation of TB sputum sample: The challenges

India sharps lack in adequate number of accredited laboratories for testing tuberculosis patients in the country that makes sufficient and elaborate testing for the pathogen challenging and difficult. This includes all form of testing, *viz,* TB culture, detection and drug resistant testing. Out of many reasons that plague the issue, one key cause is transportation of sputum samples. Across the world, sputum is collected in glass or plastic containers with a cap and transportation is most often done at room temperature. The transportation system of sputum in countries such as India is highly unorganized and hence it often takes days to weeks before samples reach from rural India to any centralized TB testing facility in the country for advanced TB testing. This ultimately results in high level of contamination of sputum and loss of positive cultures when detected in most formats of testing [222, 223, 224]. Transportation of TB sputum samples from peripheral sites to centralized facilities is also a costly affair mainly for the need for maintaining cold chain during the process.

8.3. The need for a novel method of TB sputum handling system

These problems call for a new look into the age-old method of TB sputum collection, transportation and extraction of DNA for NA-based testing. There is a large unmet need for improving this age-old method of handling sputum specially for DNA-based testing and demonstrating the solution on a large population to establish its efficacy and suitability. In this context recent innovations by way of developing reagent coated cards for long term archival of sputum borne DNA is noteworthy [312]. One such product named the TBSend card has been demonstrated to align with cartridge based nucleic acid testing (CB NAAT) platform such as geneXpert and has acceptable sensitivity [313]. Further, this device is biosafe when sputum is spotted onto it and can be stored for over 6 years at room temperature with no loss of archived DNA [312].

8.4. Extraction of *M. tuberculosis* DNA

The microbiome resident in the respiratory environment are areas of interest in recent times due to the spreading problem of chronic respiratory infections [225]. Not just for infections but also for disorders such as chronic obstructive pulmonary disease (COPD) and bronchiectasis which include patients suffering from cystic fibrosis, sputum are invaluable samples for disease investigation [226, 227, 228, 229]. Sputum is a comparatively difficult clinical sample owing to its complex biological matrix. Hence a wide range of pretreatment protocols evolved over the time that include extensive use of solubilizing agents such as dithiothreitol [230]. The protocol for sputum DNA extraction using solid phase purification method exploits the use of silica membrane-loaded spin columns and a strong centrifugal force [231]. This method as well as those that use glass powder, diatomaceous earth and various anion exchange carriers are the popular method of extraction of nucleic acid from such clinical specimens. In almost all cases, the key steps involved are the lysis of cells, the adsorption of DNA onto the membrane, the cleaning of the membrane using various wash buffers followed be elution of the DNA [232].

Molecular biology and relate technologies offer the unique advantage of performing microbial diagnostics without going through the cumbersome process of bacterial culture which is not only expensive but also time consuming. In cases of human disease when biopsy based clinical samples are involved, irrespective of the method that is employed for extraction of a bacterial pathogen DNA, the extracted analyte is almost always a mixture of host and pathogen DNA intermingled together [233]. Extraction of bacterial DNA from human tissue samples is indeed a challenge and hence quite understandingly, there are extremely few if any, discrete kit or set of reagents available that is exclusively dedicated to such extraction of microbial pathogen DNA from human tissue. A wide spectrum and variety of methods are hence reported for such extraction of human microbial pathogen DNA. This includes enrichment of nuclei for differential isolation of microbes [234], various in-house DNA extractions [235, 236, 237, 238, 239], a range of commercial kits [240] and use of several of these platforms together in home brew format [241, 242]. Absence of a perfect and globally standardized method has raised the possibility of generation of false negatives particularly when Polymerase Chain Reaction is used for detection, where (lack or presence of) inhibitors play a crucial role in the detection process [243]. This issue is the primary reason for the use of internal control amplification strategy. In this strategy a known DNA apart from the pathogen to be detected, is targeted using primers and probes in a polymerase chain reaction. Successful and simultaneous amplification of this "other" DNA indicates absence of an inhibitor that often get carried over during the extraction process. This internal control-target can be a gene segment of the host DNA [244], a synthetic, laboratory-cultured, pure plasmid DNA [244, 245] or any other, unrelated bacterial DNA that is externally added to the reaction [246].

It is important to note that while an internal control reaction using an alternative and unrelated amplification target can successfully indicate the status of inhibition during a thermal cycling process, it is in no way a guarantee that the pathogen DNA, originating from cells which are deeply embedded inside the tissue, will also be successfully detected. This is particularly important for *M. tuberculosis* for several reasons. *M. tuberculosis* cells grown in synthetic medium in the laboratory has extremely complex cell wall composition [237, 238, 239]. Pathogenic bacterial cells that are residing within a phagosome, within a host cell or inside a granuloma require a range of extraction reaction steps to successfully extract the DNA. If these special steps are not applied, the release of DNA from the pathogen microbes will be suboptimal. Yet another cause for very low target pathogen DNA is overall low bacterial burden in cases of paucibacillary diseases such as tuberculoid leprosy. These are few of the prime reasons for the development of a range of methods for extraction of DNA from microbes with special reference to human tissue as the source.

Real Time PCR is one of the most popular methods of detection of *M. tuberculosis* DNA. This is due to the fact that this technique is accurate, sensitive and fast. It is widely agreed that tuberculosis being a health problem prevalent worldwide, its early detection is essential for optimal control of the disease [250, 252, 253]. Real Time PCR has the capability to not only detect the pathogen but also detect its drug resistant variants when DNA is extracted from clinical samples originating from tuberculosis patients [247, 249, 251, 262, 263, 264, 265]. However, this calls for high quality

extraction of DNA from the clinical samples. For this reason, several strategies are employed. This includes use of proteolytic enzymes such as proteinase K, detergents, heat treatment and combination of these agents [247, 248, 251, 254, 256, 257, 258, 259, 260, 261, 263, 266]. One of the most popular techniques for lysis of *M. tuberculosis* cells during the process of DNA extraction is heating infected sputum in presence of Tris chloride and EDTA [256]. A range of commercial kits are also available that generate acceptable quality of *M. tuberculosis* DNA. These are the PrepMan ultra extraction from Applied Biosystems, Inc., Foster City, CA, bacterial cell lysis extraction method from Infection Diagnostics, Inc. Quebec, Canada, QIAmp DNA mini kit manufactured by QIAGEN, Inc., Valencia, CA, a sodium dodecyl sulfate (SDS) and Triton X mixture-based DNA extraction process [255] and use of the method of sonication in the presence of SDS and Triton X 100.

During the past several years, several national programs aimed to popularize *M. tuberculosis* drug sensitivity testing (DST) program for improved surveillance in low- and medium-income countries faced severe challenges. This include the high infrastructural cost of performing DST with proper biocontainment facilities that is mandatory for detection of *M. tuberculosis* complex members using culture and other phenotypic DST techniques (pDST). In current times, for detection of drug resistant tuberculosis, molecular probes are employed. This includes genotypic drug sensitivity testing or genotypic DST such as GeneXpert MTB/RIF and Hain Line Probe Assay (LPA). The other popular method of DST is the culture method that include MGIT liquid culture or LJ solid culture. However, these methods are laborious and time consuming [267, 268, 269, 270]. The potential advantage of use of molecular probes coupled with high-end engineering efforts to automate processes have made detection of Tuberculosis by MTB/RIF and Xpert MTB/RIF Ultra assays (GeneXpert (Cepheid, Sunnyvale, CA, USA),) feasible and possible. They are operated using automated machines that provide data in sample-to-result format. But, the Xpert assay, particularly the older version of it is not comprehensive and does not cover the entire spectrum of drug resistant *M. tuberculosis* mutations. Instead, it covers selected genes and their mutations for predicting *M. tuberculosis* drug resistance. Given the fact that this has important considerations from the point of view of treatment, another test has eventually come into vogue. The GenoType MTB DR plus and the MTB DR manufactured by Hain Life science, Germany provides a viable alternative by covering almost all relevant mutations within the *M. tuberculosis* genome that are related to drug resistant tuberculosis.

In recent years, there is an increased interest in high end sequencing-based *M. tuberculosis* drug sensitivity testing in both low as well as high tuberculosis-burden countries. This has led to the popularity of next generation sequencing as a potent technology to provide extensive coverage of drug resistant mutations within *M. tuberculosis* genome. Apart from the high cost and requirement for high end infrastructure, the other most important hurdle in further popularizing this technology is the need for high quality pathogen DNA. This therefore calls for prior extraction of DNA from the clinical sample after culturing it in a *M. tuberculosis* specific medium [271, 272]. The reason for this apparent handicap is the contamination of the pathogen DNA with host nucleic acid which get co-extracted when the clinical sample is subjected to direct nucleic acid extraction. However, when the clinical sample is subjected to culture using medium that exclusively support *M. tuberculosis* growth, most of the contaminating cells die leaving behind pure *M tuberculosis* cells which are then used for extraction of DNA. In this context it is worth noting that when clinical samples infected with *M. tuberculosis* is cultured then only those variants or species that respond to culture are represented in the grown culture and hence is not a true representative of the actual microbial flora of *M. tuberculosis* present in the clinical sample [272, 273, 274].

8.5. Molecular detection of *M. tuberculosis*

A good treatment demands quick and accurate detection of pathogen along with its response-behavior towards crucial drugs. Further, the need for its adaptability to resource limited setting also hold a crucial role with regard to its usefulness in countries such as India. [275]. The process of culture-based detection has now become rapid and more efficient. Techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has evolved for accurate identification of the pathogen.

Rapid advancement in the molecular detection technologies has dramatically reduced the time for detection of tuberculosis pathogen from weeks to hours. [276, 277]. A majority of protocols detect the DNA or RNA of this pathogen by various amplification technologies and also determine its drug susceptibility by either amplification or hybridization-based methods [278].

The National Rural Health Mission [NRHM] was started in the year 2005 in India with a special aim of providing quality healthcare to rural population of India [279]. The basic aim of this movement still holds true for control of tuberculosis in this country. Available technologies are lesser in number when economical and accurate Tb detection methods are considered that are easily adaptable to rural settings of the country. In this scenario, focus into newer methods of

detection of *M. tuberculosis* that can be decentralized to village level and can adapt itself to resource limited settings is the need of the hour.

The process of polymerase chain reaction or PCR that is now widely acknowledged as the backbone of molecular biology, is one of the most sensitive and accurate method of detecting *M. tuberculosis* using its genome as the analyte. This is particularly useful when the quantum of microbe is low and hence require an ultra-high degree of sensitivity [280]. For *M. tuberculosis*, the most popular and effective DNA marker is the IS61110 insertional element that is present in the *M. tuberculosis* genome in multiple copies. More importantly, this target is found exclusively within the *M tuberculosis* complex thus aiding in specificity towards the species that is responsible for the disease [281].

In the diagnostics of *M. tuberculosis* there are frequent variabilities encountered which are mostly due to variation in the quality and quantity of DNA extracted from clinical samples [282]. In order to incorporate molecular diagnostics of tuberculosis in routine diagnostic protocol for infectious diseases, it needs to be simple, highly effective and with steps that can effectively remove inhibitor molecules that can retard or stop the process of PCR.

In the past few decades, the tuberculosis diagnostics community has seen the development of a method that is internationally standardized and intended for generating DNA fingerprint of *M tuberculosis* [283]. This technology has allowed the scientific community to get greater insights into the dynamics of tuberculosis epidemics and allowed comparison of different strains of the pathogens that spread during onset of the disease in a population in the world [284, 285].

However, one of the side effects of this world-wide focus on understanding the *M. tuberculosis* genome was large scale extraction of DNA from the pathogen in all the laboratories working in this domain. This essentially raise major biosafety issues since it was not unequivocally proven that the pathogen when heat killed, was biosafe for the person who is processing the clinical samples. It was believed by a section of the scientific community that the pathogen may remain live and active when placed within the interphase region of the organic extraction tube thus rendering the operator of the process suspectable to contracting the disease [286]. The simplest and most practical solution for this problem was the suggestion to use a biosafety level 3 facility. This facility has provision to adequately protect an operator from pathogen infection However, setting up such a facility in all the laboratories was not only expensive but impractical owing to the sophistication involved in the infrastructure. [286].

Since a decade, the field of *M. tuberculosis* has seen dramatic development of newer molecular diagnostic tests. Colloquially referred to as the Nucleic Acid Amplification Tests or the NAATs, these tests use amplification of its intended genome target as one of the first steps towards detection. These technologies have disrupted the *M. tuberculosis* diagnostics with low cost, and highly efficient solution for detection of the pathogen [287, 288]. Several of these technologies, including the ubiquitous polymerase chain reaction-based ones, are currently available in the market.

The Line probe assays (LPA) are one of the most popular *M. tuberculosis* diagnostic tests that detect resistance towards the first-line antituberculosis drugs (for MDR-TB) [289]. The tests include the GenoType MTBDRplus (Hain Lifesciences-Bruker, Nehren, Germany) and the Nipro NTM_MDRTB II (manufactured by Osaka, Japan)]. Presently, more sophisticated version of the line probe assay has arrived in the market (GenoType MTBDRsl version 2.0; Hain Lifesciences-Bruker) that can detect mutations conferring resistance to amikacin, kanamycin and capreomycin. Therefore, this test is widely recommended by clinicians for taking important tuberculosis-related therapeutic decisions [289].

Yet another technology that is used for detection of *M. tuberculosis* is the Loop-mediated isothermal amplification. This test requires minimal infrastructural need [290] and can be adapted in peripheral settings of the country. The technology was developed by Eiken Chemical Company located at Tokyo, Japan and has been recommended by the World Health Organization (WHO [291]) as a potent substitute for smear microscopy because of its high level of diagnostic accuracy compared to smear microscopy methods. However, despite its apparent advantages, this technology still remains under-utilized by the global *M. tuberculosis* diagnostic community [292].

In the year 2010, the world health Organization officially approved the use of Xpert MTB/RIF (Cepheid, Sunnyvale) [293] as a real time PCR based test for detecting *M tuberculosis* and its drug resistant variants based in mutations present in the *rpo*B gene. In the year 2017, WHO further recommended Xpert Ultra (Cepheid) (Ultra), an improved version of the earlier test with larger coverage of drug resistant genes and mutations. It was recommended for children & adults irrespective of their HIV-1 co-infection status and was suggested for use over smear microscopy and culture [294].

Yet another technology that is effective for diagnostics of *M. tuberculosis* is known as the Simultaneous amplification and testing method [295, 296]. Extensive studies have demonstrated that the efficacy for detection of *M. tuberculosis* by this method is cent per cent [297, 298]. This suggest that the assay platform has huge potential for clinicians involved in treatment of tuberculosis. Further to this, this technology uses *M. tuberculosis* RNA instead of DNA as the analyte. Since presence of RNA in an organism is indicative of its living status, it is concluded that this technology detects live *M. tuberculosis* bacilli in clinical sample.

One of the hallmarks of modern DNA based drug sensitivity testing platforms for *M. tuberculosis* is the drastic reduction in time required to get the results. This is in stark difference to the DST by culture method where the turnaround time for getting the results is long.

In the year 1970, DNA sequencing evolved as a potent platform for determining the nucleotide sequences of DNA fragments [301]. However, the technology lacked desired high throughput and had limitations by way of cost incurred per gene that was analyzed. This led to the development of the next generation sequencing (NGS) or the massive parallel sequencing technique [302]. This technology, although expensive, now plays a key role in epidemiological investigations of tuberculosis. *M. tuberculosis* drug susceptibility testing. This issue is now well covered by the NGS technology platform *albeit* with some recognized disadvantages that is discussed elsewhere [303, 304, 305].

Apart from NAAT tests that address DST in *M. tuberculosis*, another technology platform that provides result in a very short period of time is flow cytometry. This technology platform is very promising and is often labelled as one of the best choices for rapid detection as well as quantification of a large number of microbes present in the environment, in food substances and also in human clinical samples [299, 300]. In the year 1995, Norden and his coworkers [306] first described flow cytometry as a quick test for determining drug susceptibility in *M. tuberculosis*. They employed the chemical Fluorescein Diacetate and tested *M. tuberculosis* strain H37Ra that is known to be susceptible to all anti tuberculosis drugs and substantiated the reports of other authors working in the domain of *M. tuberculosis* drug resistance testing [307, 308]. Although flow cytometry is proven to be a rapid and useful test, its operations raised significant biosafety concerns which led to the recommendation that samples should be treated with paraformaldehyde prior to flow cytometry analysis [309].

Another variant of the flow cytometry method of testing *M. tuberculosis* drug susceptibility is the use of SYTO 9 stain and prior heating of the clinical samples to kill the active and living *M. tuberculosis* bacilli. This addressed the much-required biosafety concern related to this platform. SYTO dye has the property to bind to microbial DNA leading to marked enhancement of its fluorescence. For this test, samples were treated with different drugs and latter screened with the SYTO dye. Those bacilli that were susceptible to a drug got killed allowing the SYTO dye to stain its genome and fluoresce [310]. With the course of time the dye was replaced with fluorescent nanoparticles which has fluorescent properties and were far superior compared to conventional dyes. Another version of this test platform used the SYTO 9 dye, propidium iodide and ethidium monoazide and effectively discriminated between live, drug injured and dead *M. tuberculosis* bacilli. [311].

The evolution of the techniques of DNA and RNA extraction from biological cells is one of the most significant milestones in the long path of development of molecular diagnostics. The availability of clean and sufficient DNA provoked the scientific community to develop sensitive and automated detection platforms. These innovations demonstrated wonder harmony between chemistry and mechanics. Their developments benefitted a large section of the diseased community and ambitious plans such as eradication of tuberculosis from India as much as from the world can only be made due to the unparallel development of nucleic acid extraction and its detection technology that took place over the years.

9. Conclusion

Extraction of nucleic acids from a wide range of biological sources is now an integral part of all research laboratories across the world that are involved in molecular diagnostic research of genetic and infectious diseases. This review provides a glimpse at the technologies that evolved with time to extract pure DNA and RNA from challenging biological sources which formed the basis for development of complex and sophisticated detection technologies in a wider spectrum of analytical platforms. A special reference has been made to the extraction of nucleic acid as an analyte for *Mycobacterium tuberculosis* and different detection methods available for detecting it. This narration provides a concise description of few of the fundamental aspects of human molecular diagnostics with special reference to tuberculosis, a life-threatening disease that has infected an estimated number of 10.6 million people worldwide in the year 2022 that include 5.8 million men, 3.5 million women, and a high of 1.3 million children across the globe as per the World Health Organization (WHO) (https://www.**who**.int/news-room/fact-sheets/detail/tuberculosis).

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Pham, X. H., Baek, A., Kim, T. H., Lee, S. H., Rho, W. Y., Chung, W. J., Kim, D. E., & Jun, B. H. (2017). Graphene Oxide Conjugated Magnetic Beads for RNA Extraction. Chemistry, an Asian journal, 12(15), 1883–1888.

[2] Mosley O, Melling L, Tarn MD, Kemp C, Esfahani MMN, Pamme N(2016). Sample introduction interface for on-chip nucleic acid-based analysis of Helicobacter pylori from stool samples. Lab Chip, 16:2108–15

[3] Kamat, V., Pandey, S., Paknikar, K., & Bodas, D. (2018). A facile one-step method for cell lysis and DNA extraction of waterborne pathogens using a microchip. Biosensors and Bioelectronics, 99, 62-69.

[4] Mullegama SV., Alberti MO., Au C., Li Y., Toy T., Tomasian V., Xian RR. (2019), Nucleic Acid Extraction from Human Biological Samples. Methods Mol Biol., 1897, 359-383.

[5] S. C. Tan, B. C. Yiap. (2009), DNA, RNA, and protein extraction: the past and the present. Journal of Biomedicine and Biotechnology, 2009, 574398.1-10.

[6] Wink M.(2006), An Introduction to Molecular Biotechnology: Molecular Fundamentals, Methods and Application in Modern Biotechnology. Weinheim, Germany: Wiley-VCH.

[7] Doyle, K. (1996). The source of discovery: protocols and applications guide. Madison: PROMEGA. Madison, Wis, USA.

[8] Buckingham, L. (2019). Molecular diagnostics: fundamentals, methods and clinical applications. FA Davis. Philadelphia, Pa, USA.

[9] Cseke, L.J., Kaufman, P.B., Podila, G.K., & Tsai, C.-J. (Eds.). (2004). Handbook of Molecular and Cellular Methods in Biology and Medicine (2nd ed.). CRC Press, Boca Raton, Fla, USA.

[10] Brooks, G. (1998) Biotechnology in healthcare: an introduction to biopharmaceuticals. The Pharmaceutical Press, London, UK.

[11] Ali N, Rampazzo RCP, Costa ADT, Krieger MA. (2017), Current Nucleic Acid Extraction Methods and Their Implications to Point-of-Care Diagnostics, BioMed Research International., 2017, 1-13.

[12] M. Lesk. (1969), Why does DNA contain thymine and RNA uracil? Journal of Theoretical Biology, 22(3), 537–540.

[13] Poh, J. J., & Gan, S. K. (2014). Comparison of customized spin-column and salt-precipitation finger-prick blood DNA extraction. Bioscience reports, 34(5), e00145.

[14] Aggarwal S. Phadke SR. (2015), Medical genetics and genomic medicine in India: current status and opportunities ahead., Mol Genet Genomic Med., 3(3), 160-171.

[15] Rao GG., Agarwal A., Batura D. (2020), Testing times in Coronavirus disease (COVID-19): A tale of two nations. Med J Armed Forces India, 76(3), 243-249.

[16] S. V. Smarason and A. V. Smith. (2003), Method for desalting nucleic acids, "United State patent US 2003/0186247 A1, deCODE genetics ehf., October. 1-11.

[17] J. Sambrook and D. Russel (2001), Molecular Cloning: A Laboratory Manual.Cold Spring Harbor Laboratory Press, 3.

[18] Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W. J., & Goodman, H. M. (1977). Rat insulin genes: construction of plasmids containing the coding sequences. Science, 196(4296), 1313-1319.

[19] Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry, 162(1), 156-159.

[20] H. C. Birnboim and J. Doly (1979), A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research, 7(6), 1513–1523.

[21] Yamamoto S., Kitagawa W., Nakano M., Asakura H., Iwabuchi E., Sone T., Asano K. (2020), Plasmid Sequences of Four Large Plasmids Carrying Antimicrobial Resistance Genes in Escherichia coli Strains Isolated from Beef Cattle in Japan. Microbiol Resour Announc.2020, 14, 9(20), e00219-20.

[22] Chakraborty S., Saha A., Neelavar Ananthram A. (2020), Comparison of DNA extraction methods for non-marine molluscs: is modified CTAB DNA extraction method more efficient than DNA extraction kits? 3 Biotech., 10(2), 2051-2057.

[23] Xu M., Bailey MJ., Look J., Baneyx F. (2020), Affinity purification of Car9-tagged proteins on silica-derivatized spin columns and 96-well plates. Protein Expr Purif., 170, 105608-105633.

[24] Lee H., Park C., Na W., Park KH., Shin S. (2020), Precision cell-free DNA extraction for liquid biopsy by integrated microfluidics. NPJ Precis Oncol., 4(3), 1-10.

[25] Asencio C., Chatterjee A., Hentze MW. (2018), Silica-based solid-phase extraction of cross- linked nucleic acid-bound proteins., Life Science Alliance, 1(3), 1-8.

[26] Fidler G., Tolnai E., Stagel A., Remenyik J., Stundl L., Gal F., Biro S., Paholcsek M. (2020), Tendentious effects of automated and manual metagenomic DNA purification protocols on broiler gut micro- biome taxonomic profiling. Scientific Reports, 10(1), 3419.1-16.

[27] Pearlman SI., Leelawong M., Richardson KA., Adams NM., Russ PK., Pask ME., Wolfe AE., Wessely C., Haselton FR. (2020), Low-Resource Nucleic Acid Extraction Method Enabled by High-Gradient Magnetic Separation. ACS Appl Mater Interfaces., 12(11), 12457-12467.

[28] Pham, XH., Hahm E., Kim HM., Son BS., Jo A., An J., Tran Thi TA., Nguyen DQ., Jun BH. (2020), Silica-Coated Magnetic Iron Oxide Nanoparticles Grafted onto Graphene Oxide for Protein Isolation. Nanomaterials, 10, 117-131.

[29] Shi R., Lewis RS., Panthee DR. (2018), Filter paper-based spin column method for cost- efficient DNA or RNA purification. PLoS One., 13(12), e0203011-25.

[30] Sakmann, B., & Neher, E. (1984). Patch clamp techniques for studying ionic channels in excitable membranes. Annual review of physiology, 46, 455–472.

[31] Goodfellow, M.; Stackebrandt, E. (1991) Nucleic Acid Techniques in Bacterial Systematics; John Wiley & Sons: Hoboken, NJ, USA, Volume 5.

[32] Harrison S. T. (1991). Bacterial cell disruption: a key unit operation in the recovery of intracellular products. Biotechnology advances, 9(2), 217–240.

[33] Markets and Markets™ Research Private Ltd. Cell Lysis/Cell Fractionation Market-Global Forecasts to 2021. Available online: http://www.marketsandmarkets.com/Market-Reports/cell-lysis-market 260138321. html (accessed on 6 March 2017).

[34] Mark, D., Haeberle, S., Roth, G., von Stetten, F., & Zengerle, R. (2010). Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. Chemical Society reviews, 39(3), 1153–1182.

[35] Lubiński, J., Górski, B., Kurzawski, G., Jakubowska, A., Cybulski, C., Suchy, J., Dêbniak, T., Grabowska, E., Lener, M. and Nej, K. (2002). Molecular basis of inherited predispositions for tumors. Acta Biochimica Polonica, 49(3), 571-581.

[36] Kurzawski, G., Dymerska, D., Serrano-Fernández, P., Trubicka, J., Masojć, B., Jakubowska, A., & Scott, R. J. (2012). DNA and RNA analyses in detection of genetic predisposition to cancer. Hereditary cancer in clinical practice, 10(1), 17.

[37] Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., & Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Sciences of the United States of America, 86(8), 2766–2770.

[38] Nagamine, C. M., Chan, K., & Lau, Y. F. (1989). A PCR artifact: generation of heteroduplexes. American journal of human genetics, 45(2), 337–339.

[39] Cotton, R. G., Rodrigues, N. R., & Campbell, R. D. (1988). Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proceedings of the National Academy of Sciences of the United States of America, 85(12), 4397–4401.

[40] O'Donovan, M. C., Oefner, P. J., Roberts, S. C., Austin, J., Hoogendoorn, B., Guy, C., Speight, G., Upadhyaya, M., Sommer, S. S., & McGuffin, P. (1998). Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics, 52(1), 44–49.

[41] Myers, R. M., Maniatis, T., & Lerman, L. S. (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods in enzymology, 155, 501–527.

[42] Liu, W., Smith, D. I., Rechtzigel, K. J., Thibodeau, S. N., & James, C. D. (1998). Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic acids research, 26(6), 1396–1400.

[43] Jones, A. C., Austin, J., Hansen, N., Hoogendoorn, B., Oefner, P. J., Cheadle, J. P., & O'Donovan, M. C. (1999). Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. Clinical chemistry, 45(8 Pt 1), 1133–1140.

[44] Arnold, N., Gross, E., Schwarz-Boeger, U., Pfisterer, J., Jonat, W., & Kiechle, M. (1999). A highly sensitive, fast, and economical technique for mutation analysis in hereditary breast and ovarian cancers. Human mutation, 14(4), 333–339.

[45] Gross, E., Arnold, N., Goette, J., Schwarz-Boeger, U., & Kiechle, M. (1999). A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. Human genetics, 105(1-2), 72–78.

[46] Xiao, W., & Oefner, P. J. (2001). Denaturing high-performance liquid chromatography: A review. Human mutation, 17(6), 439–474.

[47] Kurzawski, G., Safranow, K., Suchy, J., Chlubek, D., Scott, R. J., & Lubiński, J. (2002). Mutation analysis of MLH1 and MSH2 genes performed by denaturing high-performance liquid chromatography. Journal of biochemical and biophysical methods, 51(1), 89–100.

[48] Rosenthal, A., & Charnock-Jones, D. S. (1992). New protocols for DNA sequencing with dye terminators. DNA sequence: the journal of DNA sequencing and mapping, 3(1), 61–64.

[49] Glenn T. C. (2011). Field guide to next-generation DNA sequencers. Molecular ecology resources, 11(5), 759–769.

[50] Walsh, T., Lee, M. K., Casadei, S., Thornton, A. M., Stray, S. M., Pennil, C., Nord, A. S., Mandell, J. B., Swisher, E. M., & King, M. C. (2010). Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. Proceedings of the National Academy of Sciences of the United States of America, 107(28), 12629–12633.

[51] Walsh, T., Casadei, S., Lee, M. K., Pennil, C. C., Nord, A. S., Thornton, A. M., Roeb, W., Agnew, K. J., Stray, S. M., Wickramanayake, A., Norquist, B., Pennington, K. P., Garcia, R. L., King, M. C., & Swisher, E. M. (2011). Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. Proceedings of the National Academy of Sciences of the United States of America, 108(44), 18032–18037.

[52] Pritchard, C. C., Smith, C., Salipante, S. J., Lee, M. K., Thornton, A. M., Nord, A. S., Gulden, C., Kupfer, S. S., Swisher, E. M., Bennett, R. L., Novetsky, A. P., Jarvik, G. P., Olopade, O. I., Goodfellow, P. J., King, M. C., Tait, J. F., & Walsh, T. (2012). ColoSeq provides comprehensive lynch and polyposis syndrome mutational analysis using massively parallel sequencing. The Journal of molecular diagnostics : JMD, 14(4), 357–366.

[53] Yates, L. R., Seoane, J., Le Tourneau, C., Siu, L. L., Marais, R., Michiels, S., Soria, J. C., Campbell, P., Normanno, N., Scarpa, A., Reis-Filho, J. S., Rodon, J., Swanton, C., & Andre, F. (2018). The European Society for Medical Oncology (ESMO) Precision Medicine Glossary. Annals of oncology : official journal of the European Society for Medical Oncology, 29(1), 30–35.

[54] Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., & Knippers, R. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer research, 61(4), 1659–1665.

[55] Suzuki, N., Kamataki, A., Yamaki, J., & Homma, Y. (2008). Characterization of circulating DNA in healthy human plasma. Clinica chimica acta; international journal of clinical chemistry, 387(1-2), 55–58.

[56] Johann, D. J., Jr, Steliga, M., Shin, I. J., Yoon, D., Arnaoutakis, K., Hutchins, L., Liu, M., Liem, J., Walker, K., Pereira, A., Yang, M., Jeffus, S. K., Peterson, E., & Xu, J. (2018). Liquid biopsy and its role in an advanced clinical trial for lung cancer. Experimental biology and medicine (Maywood, N.J.), 243(3), 262–271.

[57] Tamminga, S., van Maarle, M., Henneman, L., Oudejans, C. B., Cornel, M. C., & Sistermans, E. A. (2016). Maternal Plasma DNA and RNA Sequencing for Prenatal Testing. Advances in clinical chemistry, 74, 63–102.

[58] Sidransky D. (1997). Nucleic acid-based methods for the detection of cancer. Science (New York, N.Y.), 278(5340), 1054–1059.

[59] Ziegler, A., Koch, A., Krockenberger, K., & Grosshennig, A. (2012). Personalized medicine using DNA biomarkers: a review. Human genetics, 131(10), 1627–1638.

[60] Schwarzenbach, H., Hoon, D. S., & Pantel, K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. Nature reviews. Cancer, 11(6), 426–437.

[61] Diehl, F., Schmidt, K., Choti, M. A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S. A., Kinzler, K. W., Vogelstein, B., & Diaz, L. A., Jr (2008). Circulating mutant DNA to assess tumor dynamics. Nature medicine, 14(9), 985–990.

[62] Crowley, E., Di Nicolantonio, F., Loupakis, F., & Bardelli, A. (2013). Liquid biopsy: monitoring cancer-genetics in the blood. Nature reviews. Clinical oncology, 10(8), 472–484.

[63] Wan, J. C. M., Massie, C., Garcia-Corbacho, J., Mouliere, F., Brenton, J. D., Caldas, C., Pacey, S., Baird, R., & Rosenfeld, N. (2017). Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nature reviews. Cancer, 17(4), 223–238.

[64] Gerlinger, M., Rowan, A. J., Horswell, S., Math, M., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., Varela, I., Phillimore, B., Begum, S., McDonald, N. Q., Butler, A., Jones, D., Raine, K., Latimer, C., Santos, C. R., … Swanton, C. (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England journal of medicine, 366(10), 883–892.

[65] Oltedal, S., Aasprong, O. G., Møller, J. H., Kørner, H., Gilje, B., Tjensvoll, K., Birkemeyer, E. M., Heikkilä, R., Smaaland, R., & Nordgård, O. (2011). Heterogeneous distribution of K-ras mutations in primary colon carcinomas: implications for EGFR-directed therapy. International journal of colorectal disease, 26(10), 1271–1277.

[66] Kim, M. J., Lee, H. S., Kim, J. H., Kim, Y. J., Kwon, J. H., Lee, J. O., Bang, S. M., Park, K. U., Kim, D. W., Kang, S. B., Kim, J. S., Lee, J. S., & Lee, K. W. (2012). Different metastatic pattern according to the KRAS mutational status and site-specific discordance of KRAS status in patients with colorectal cancer. BMC cancer, 12, 347.

[67] Fleischhacker, M., & Schmidt, B. (2007). Circulating nucleic acids (CNAs) and cancer--a survey. Biochimica et biophysica acta, 1775(1), 181–232.

[68] Perkins, G., Yap, T. A., Pope, L., Cassidy, A. M., Dukes, J. P., Riisnaes, R., Massard, C., Cassier, P. A., Miranda, S., Clark, J., Denholm, K. A., Thway, K., Gonzalez De Castro, D., Attard, G., Molife, L. R., Kaye, S. B., Banerji, U., & de Bono, J. S. (2012). Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. PLoS one, 7(11), e47020.

[69] Allen, D., Butt, A., Cahill, D., Wheeler, M., Popert, R., & Swaminathan, R. (2004). Role of cell-free plasma DNA as a diagnostic marker for prostate cancer. Annals of the New York Academy of Sciences, 1022, 76–80.

[70] Chun, F. K., Müller, I., Lange, I., Friedrich, M. G., Erbersdobler, A., Karakiewicz, P. I., Graefen, M., Pantel, K., Huland, H., & Schwarzenbach, H. (2006). Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. BJU international, 98(3), 544–548.

[71] Schwarzenbach, H., Stoehlmacher, J., Pantel, K., & Goekkurt, E. (2008). Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Annals of the New York Academy of Sciences, 1137, 190–196.

[72] Sunami, E., Vu, A. T., Nguyen, S. L., Giuliano, A. E., & Hoon, D. S. (2008). Quantification of LINE1 in circulating DNA as a molecular biomarker of breast cancer. Annals of the New York Academy of Sciences, 1137, 171–174.

[73] Forshew, T., Murtaza, M., Parkinson, C., Gale, D., Tsui, D. W., Kaper, F., Dawson, S. J., Piskorz, A. M., Jimenez-Linan, M., Bentley, D., Hadfield, J., May, A. P., Caldas, C., Brenton, J. D., & Rosenfeld, N. (2012). Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science translational medicine, 4(136), 136ra68.

[74] Leary, R. J., Sausen, M., Kinde, I., Papadopoulos, N., Carpten, J. D., Craig, D., O'Shaughnessy, J., Kinzler, K. W., Parmigiani, G., Vogelstein, B., Diaz, L. A., Jr, & Velculescu, V. E. (2012). Detection of chromosomal alterations in

the circulation of cancer patients with whole-genome sequencing. Science translational medicine, 4(162), 162ra154.

[75] Misale, S., Yaeger, R., Hobor, S., Scala, E., Janakiraman, M., Liska, D., Valtorta, E., Schiavo, R., Buscarino, M., Siravegna, G., Bencardino, K., Cercek, A., Chen, C. T., Veronese, S., Zanon, C., Sartore-Bianchi, A., Gambacorta, M., Gallicchio, M., Vakiani, E., Boscaro, V., … Bardelli, A. (2012). Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature, 486(7404), 532–536.

[76] Chan, K. C., Jiang, P., Zheng, Y. W., Liao, G. J., Sun, H., Wong, J., Siu, S. S., Chan, W. C., Chan, S. L., Chan, A. T., Lai, P. B., Chiu, R. W., & Lo, Y. M. (2013). Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clinical chemistry, 59(1), 211–224.

[77] Yao, W., Mei, C., Nan, X., & Hui, L. (2016). Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. Gene, 590(1), 142–148.

[78] Price, C. W., Leslie, D. C., & Landers, J. P. (2009). Nucleic acid extraction techniques and application to the microchip. Lab on a chip, 9(17), 2484–2494.

[79] Devonshire, A. S., Whale, A. S., Gutteridge, A., Jones, G., Cowen, S., Foy, C. A., & Huggett, J. F. (2014). Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Analytical and bioanalytical chemistry, 406(26), 6499–6512.

[80] Mauger, F., Dulary, C., Daviaud, C., Deleuze, J. F., & Tost, J. (2015). Comprehensive evaluation of methods to isolate, quantify, and characterize circulating cell-free DNA from small volumes of plasma. Analytical and bioanalytical chemistry, 407(22), 6873–6878.

[81] Sherwood, J. L., Corcoran, C., Brown, H., Sharpe, A. D., Musilova, M., & Kohlmann, A. (2016). Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC). PloS one, 11(2), e0150197.

[82] Melzak, K. A., Sherwood, C. S., Turner, R. F., & Haynes, C. A. (1996). Driving forces for DNA adsorption to silica in perchlorate solutions. Journal of colloid and interface science, 181(2), 635-644.

[83] Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M., & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. Journal of clinical microbiology, 28(3), 495–503.

[84] Tan, S. C., & Yiap, B. C. (2009). DNA, RNA, and protein extraction: the past and the present. Journal of biomedicine & biotechnology, 2009, 574398.

[85] Petralia, S., Sciuto, E. L., & Conoci, S. (2017). A novel miniaturized biofilter based on silicon micropillars for nucleic acid extraction. Analyst, 142(1), 140-146.

[86] De, A., Sparreboom, W., van den Berg, A., & Carlen, E. T. (2014). Rapid microfluidic solid-phase extraction system for hyper-methylated DNA enrichment and epigenetic analysis. Biomicrofluidics, 8(5), 054119.

[87] Min, Junhong, Joon-Ho Kim, Youngsun Lee, Kak Namkoong, Hae-Cheon Im, Han-Nah Kim, Hae-Yeong Kim, Nam Huh, and Young-Rok Kim. (2011). Functional integration of DNA purification and concentration into a real time micro-PCR chip. Lab on a Chip, 11(2), 259-265.

[88] Chen, D., Mauk, M., Qiu, X., Liu, C., Kim, J., Ramprasad, S., Ongagna, S., Abrams, W. R., Malamud, D., Corstjens, P. L., & Bau, H. H. (2010). An integrated, self-contained microfluidic cassette for isolation, amplification, and detection of nucleic acids. Biomedical microdevices, 12(4), 705–719.

[89] Qiu, X., Chen, D., Liu, C., Mauk, M. G., Kientz, T., & Bau, H. H. (2011). A portable, integrated analyzer for microfluidic - based molecular analysis. Biomedical microdevices, 13(5), 809–817.

[90] Hagan, K. A., Bienvenue, J. M., Moskaluk, C. A., & Landers, J. P. (2008). Microchip-based solid-phase purification of RNA from biological samples. Analytical chemistry, 80(22), 8453–8460.

[91] Hagan, K. A., Meier, W. L., Ferrance, J. P., & Landers, J. P. (2009). Chitosan-coated silica as a solid phase for RNA purification in a microfluidic device. Analytical chemistry, 81(13), 5249–5256.

[92] Karle, M., Miwa, J., Czilwik, G., Auwärter, V., Roth, G., Zengerle, R., & von Stetten, F. (2010). Continuous microfluidic DNA extraction using phase-transfer magnetophoresis. Lab on a chip, 10(23), 3284–3290.

[93] Lee, H., Na, W., Park, C., Park, K. H., & Shin, S. (2018). Centrifugation-free extraction of circulating nucleic acids using immiscible liquid under vacuum pressure. Scientific reports, 8(1), 5467.

[94] Jin, C. E., Koo, B., Lee, T. Y., Han, K., Lim, S. B., Park, I. J., & Shin, Y. (2018). Simple and Low-Cost Sampling of Cell-Free Nucleic Acids from Blood Plasma for Rapid and Sensitive Detection of Circulating Tumor DNA. Advanced science (Weinheim, Baden-Wurttemberg, Germany), 5(10), 1800614.

[95] Marantz, P. R., Linzer, M., Feiner, C. J., Feinstein, S. A., Kozin, A. M., & Friedland, G. H. (1987). Inability to predict diagnosis in febrile intravenous drug abusers. Annals of internal medicine, 106(6), 823–828.

[96] Samet, J. H., Shevitz, A., Fowle, J., & Singer, D. E. (1990). Hospitalization decision in febrile intravenous drug users. The American journal of medicine, 89(1), 53–57.

[97] Cosgrove, S. E., & Carmeli, Y. (2003). The impact of antimicrobial resistance on health and economic outcomes. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 36(11), 1433–1437.

[98] Pitt, T. L., & Saunders, N. A. (2000). Molecular bacteriology: a diagnostic tool for the millennium. Journal of clinical pathology, 53(1), 71–75.

[99] Naber S. P. (1994). Molecular pathology--diagnosis of infectious disease. The New England journal of medicine, 331(18), 1212–1215.

[100] Santis, G., & Evans, T. W. (1999). Molecular biology for the critical care physician. Part II: where are we now?. Critical care medicine, 27(5), 997–1003.

[101] Chamberlain, J. S., Gibbs, R. A., Ranier, J. E., Nguyen, P. N., & Caskey, C. T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic acids research, 16(23), 11141–11156.

[102] Erlich, H. A., Gelfand, D., & Sninsky, J. J. (1991). Recent advances in the polymerase chain reaction. Science (New York, N.Y.), 252(5013), 1643–1651.

[103] Power E. G. (1996). RAPD typing in microbiology--a technical review. The Journal of hospital infection, 34(4), 247–265.

[104] Higuchi, R., Dollinger, G., Walsh, P. S., & Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. Bio/technology (Nature Publishing Company), 10(4), 413–417.

[105] Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature, 449(7164), 811–818.

[106] Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America, 101(44), 15718–15723.

[107] Cebra J. J. (1999). Influences of microbiota on intestinal immune system development. The American journal of clinical nutrition, 69(5), 1046S–1051S.

[108] Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews. Immunology, 9(5), 313–323.

[109] Lai, S. K., Hida, K., Shukair, S., Wang, Y. Y., Figueiredo, A., Cone, R., Hope, T. J., & Hanes, J. (2009). Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus. Journal of virology, 83(21), 11196–11200.

[110] Taha, T. E., Hoover, D. R., Dallabetta, G. A., Kumwenda, N. I., Mtimavalye, L. A., Yang, L. P., Liomba, G. N., Broadhead, R. L., Chiphangwi, J. D., & Miotti, P. G. (1998). Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. AIDS (London, England), 12(13), 1699–1706.

[111] Watts, D. H., Fazzari, M., Minkoff, H., Hillier, S. L., Sha, B., Glesby, M., Levine, A. M., Burk, R., Palefsky, J. M., Moxley, M., Ahdieh-Grant, L., & Strickler, H. D. (2005). Effects of bacterial vaginosis and other genital infections on the natural history of human papillomavirus infection in HIV-1-infected and high-risk HIV-1-uninfected women. The Journal of infectious diseases, 191(7), 1129–1139.

[112] Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. Journal of clinical microbiology, 43(11), 5721–5732.

[113] Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., Perez-Perez, G., Blaser, M. J., & Relman, D. A. (2006). Molecular analysis of the bacterial microbiota in the human stomach. Proceedings of the National Academy of Sciences of the United States of America, 103(3), 732–737.

[114] Pei, Z., Bini, E. J., Yang, L., Zhou, M., Francois, F., & Blaser, M. J. (2004). Bacterial biota in the human distal esophagus. Proceedings of the National Academy of Sciences of the United States of America, 101(12), 4250–4255.

[115] Zhou, X., Bent, S. J., Schneider, M. G., Davis, C. C., Islam, M. R., & Forney, L. J. (2004). Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology (Reading, England), 150(Pt 8), 2565–2573.

[116] Robinson, C. J., Bohannan, B. J., & Young, V. B. (2010). From structure to function: the ecology of host-associated microbial communities. Microbiology and molecular biology reviews : MMBR, 74(3), 453–476.

[117] Ward, D. M., Weller, R., & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature, 345(6270), 63–65.

[118] Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. Science (New York, N.Y.), 308(5728), 1635–1638.

[119] Gao, Z., Tseng, C. H., Pei, Z., & Blaser, M. J. (2007). Molecular analysis of human forearm superficial skin bacterial biota. Proceedings of the National Academy of Sciences of the United States of America, 104(8), 2927–2932.

[120] Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., & Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1(Suppl 1), 4680–4687.

[121] Carrigg, C., Rice, O., Kavanagh, S., Collins, G., & O'Flaherty, V. (2007). DNA extraction method affects microbial community profiles from soils and sediment. Applied microbiology and biotechnology, 77(4), 955–964.

[122] Frostegård, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X., & Simonet, P. (1999). Quantification of bias related to the extraction of DNA directly from soils. Applied and environmental microbiology, 65(12), 5409–5420.

[123] Krsek, M., & Wellington, E. M. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. Journal of microbiological methods, 39(1), 1–16.

[124] Morgan, J. L., Darling, A. E., & Eisen, J. A. (2010). Metagenomic sequencing of an in vitro-simulated microbial community. PloS one, 5(4), e10209.

[125] Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović, M., Kekkonen, R. A., Palva, A., & de Vos, W. M. (2010). Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. Journal of microbiological methods, 81(2), 127–134.

[126] Ariefdjohan, M. W., Savaiano, D. A., & Nakatsu, C. H. (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutrition journal, 9, 23.

[127] Scupham, A. J., Jones, J. A., & Wesley, I. V. (2007). Comparison of DNA extraction methods for analysis of turkey cecal microbiota. Journal of applied microbiology, 102(2), 401–409.

[128] Inceoglu, O., Hoogwout, E. F., Hill, P., & van Elsas, J. D. (2010). Effect of DNA extraction method on the apparent microbial diversity of soil. Applied and environmental microbiology, 76(10), 3378–3382.

[129] Bürgmann, H., Pesaro, M., Widmer, F., & Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. Journal of microbiological methods, 45(1), 7–20.

[130] Forney, L. J., Zhou, X., & Brown, C. J. (2004). Molecular microbial ecology: land of the one-eyed king. Current opinion in microbiology, 7(3), 210–220.

[131] Liesack, W., Weyland, H., & Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microbial ecology, 21(1), 191–198.

[132] von Wintzingerode, F., Göbel, U. B., & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS microbiology reviews, 21(3), 213–229.

[133] Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. Nature, 449(7164), 804–810.

[134] Bertrand, H., Poly, F., Van, V. T., Lombard, N., Nalin, R., Vogel, T. M., & Simonet, P. (2005). High molecular weight DNA recovery from soils prerequisite for biotechnological metagenomic library construction. Journal of microbiological methods, 62(1), 1–11.

[135] McOrist, A. L., Jackson, M., & Bird, A. R. (2002). A comparison of five methods for extraction of bacterial DNA from human faecal samples. Journal of microbiological methods, 50(2), 131–139.

[136] Bradford P. A. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical microbiology reviews, 14(4), 933–951.

[137] Jacoby, G. A., & Bush, K. (2008). Amino Acid Sequences for TEM, SHV and OXA Extended-spectrum and inhibitor resistant ß-lactamases. Lahey Clinic. (Accessed January 1, 2008 at http://www.lahey.org/Studies/).

[138] Porteous, L. A., Armstrong, J. L., Seidler, R. J., & Watrud, L. S. (1994). An effective method to extract DNA from environmental samples for polymerase chain reaction amplification and DNA fingerprint analysis. Current microbiology, 29(5), 301–307.

[139] Herman, L. M., De Block, J. H., & Waes, G. M. (1995). A direct PCR detection method for Clostridium tyrobutyricum spores in up to 100 milliliters of raw milk. Applied and environmental microbiology, 61(12), 4141–4146.

[140] Agersborg, A., Dahl, R., & Martinez, I. (1997). Sample preparation and DNA extraction procedures for polymerase chain reaction identification of Listeria monocytogenes in seafoods. International journal of food microbiology, 35(3), 275–280.

[141] Marra, M. A., Kucaba, T. A., Hillier, L. W., & Waterston, R. H. (1999). High-throughput plasmid DNA purification for 3 cents per sample. Nucleic acids research, 27(24), e37.

[142] Skowronski, E. W., Armstrong, N., Andersen, G., Macht, M., & McCready, P. M. (2000). Magnetic, microplate-format plasmid isolation protocol for high-yield, sequencing-grade DNA. BioTechniques, 29(4), 786–792.

[143] Agarwal, A., Kumar, C., & Goel, R. (2001). Rapid extraction of DNA from diverse soils by guanidine thiocyanate method. Indian journal of experimental biology, 39(9), 906–910.

[144] Orsini, M., & Romano-Spica, V. (2001). A microwave-based method for nucleic acid isolation from environmental samples. Letters in applied microbiology, 33(1), 17–20.

[145] Tell, L. A., Foley, J., Needham, M. L., & Walker, R. L. (2003). Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three Mycobacterium spp. that affect birds. Avian diseases, 47(4), 1486–1490.

[146] Zhu, K., Jin, H., Ma, Y., Ren, Z., Xiao, C., He, Z., Zhang, F., Zhu, Q., & Wang, B. (2005). A continuous thermal lysis procedure for the large-scale preparation of plasmid DNA. Journal of biotechnology, 118(3), 257–264.

[147] Merk, S., Meyer, H., Greiser-Wilke, I., Sprague, L. D., & Neubauer, H. (2006). Detection of Burkholderia cepacia DNA from artificially infected EDTA-blood and lung tissue comparing different DNA isolation methods. Journal of veterinary medicine. B, Infectious diseases and veterinary public health, 53(6), 281–285.

[148] Lou, Y. K., Qin, H., Molodysky, E., & Morris, B. J. (1993). Simple microwave and thermal cycler boiling methods for preparation of cervicovaginal lavage cell samples prior to PCR for human papillomavirus detection. Journal of virological methods, 44(1), 77–81.

[149] Jose, J. J., & Brahmadathan, K. N. (2006). Evaluation of simplified DNA extraction methods for emm typing of group A streptococci. Indian journal of medical microbiology, 24(2), 127–130.

[150] Elkin, C. J., Richardson, P. M., Fourcade, H. M., Hammon, N. M., Pollard, M. J., Predki, P. F., Glavina, T., & Hawkins, T. L. (2001). High-throughput plasmid purification for capillary sequencing. Genome research, 11(7), 1269–1274.

[151] Dederich, D. A., Okwuonu, G., Garner, T., Denn, A., Sutton, A., Escotto, M., Martindale, A., Delgado, O., Muzny, D. M., Gibbs, R. A., & Metzker, M. L. (2002). Glass bead purification of plasmid template DNA for high throughput sequencing of mammalian genomes. Nucleic acids research, 30(7), e32.

[152] Smith, K., Diggle, M. A., & Clarke, S. C. (2003). Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. Journal of clinical microbiology, 41(6), 2440–2443.

[153] Strus M. (1997). Mechanizymy działania czynników fizycznych na drobnoustroje (Action of physical agents on microorganisms). Roczniki Panstwowego Zakladu Higieny, 48(3), 263–268.

[154] Europe Food Safety Testing Market Analysis, Trends & Forecast to 2025, With an Expected CAGR of 7.1%—ResearchAndMarkets.com. ((accessed on 4 March 2019)); Available online:

https://www.businesswire.com/news/home/20180704005223/en/Europe-Food-Safety-Testing-Market-Analysis-Trends

[155] European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. 2017, 15, e05077.

[156] Poltronieri, P., Mezzolla, V., Primiceri, E., & Maruccio, G. (2014). Biosensors for the Detection of Food Pathogens. Foods (Basel, Switzerland), 3(3), 511–526.

[157] Varga, L. (2017). The historical role of milk and dairying in shaping European societies. ´Elelmiszervizsg´alati K¨ozlem´enyek, 63, 1536–1547.

[158] IDF (International Dairy Federation). (2019). The world dairy situation 2019 – Bulletin of the IDF 501/2019. Brussels, Belgium: IDF.

[159] Hassan, A. N., & Frank, J. F. (2011). Microorganisms associated with milk. In J. W. Fuquay, P. F. Fox, & P. L. H. McSweeney (Eds.), Encyclopedia of dairy sciences (2nd ed., Vol. 3, pp. 447–457). London, UK: Academic Press.

[160] Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. FEMS Microbiology Reviews, 37, 664–698.

[161] Tonamo, A., Komlósi, I., Varga, L., Czeglédi, L., & Peles, F. (2020). Bacteriological Quality of Raw Ovine Milk from Different Sheep Farms. Animals : an open access journal from MDPI, 10(7), 1163.

[162] Franco-Duarte, R., ˇCern´akov´a, L., Kadam, S., Kaushik, K. S., Salehi, B., Bevilacqua, A., Corbo, M. R., Antolak, H., Dybka-Stępie´n, K., Leszczewicz, M., Tintino, S. R., de Souza, V. C. A., Sharifi-Rad, J., Coutinho, H. D. M., Martins, N., & Rodrigues, C. F. (2019). Advances in chemical and biological methods to identify microorganisms—from past to present. Microorganisms, 7, 130.

[163] Rajapaksha, P., Elbourne, A., Gangadoo, S., Brown, R., Cozzolino, D., & Chapman, J. (2019). A review of methods for the detection of pathogenic microorganisms. Analyst, 144(2), 396-411.

[164] De, S., Singh, R. K., Gupta, P. K., Palia, S., & Butchaiah, G. (2000). Genotyping of dairy animals using DNA from milk somatic cells. Indian Journal of Animal Sciences, 70(9), 944-946.

[165] Quigley, L., O'Sullivan, O., Beresford, T. P., Paul Ross, R., Fitzgerald, G. F., & Cotter, P. D. (2012). A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. Journal of Applied Microbiology, 113(1), 96-105.

[166] Sambrook, J., & Russell, D. W. (2006). Purification of nucleic acids by extraction with phenol:chloroform. CSH protocols, 2006(1), pdb.prot4455.

[167] Bickley, J., Short, J. K., McDowell, D. G., & Parkes, H. C. (1996). Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. Letters in applied microbiology, 22(2), 153–158.

[168] Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. Journal of applied microbiology, 113(5), 1014–1026.

[169] Kanno, C., Yamauchi, K., & Iijima, K. (1978). Solubilization of Fat Globule Membrane of Bovine Milk by Nonionic Detergents. Agricultural and Biological Chemistry, 42(12), 2299-2307.

[170] Romero, C., & Lopez-Goñi, I. (1999). Improved method for purification of bacterial DNA from bovine milk for detection of Brucella spp. by PCR. Applied and environmental microbiology, 65(8), 3735–3737.

[171] Sowmya, N., Thakur, M. S., & Manonmani, H. K. (2012). Rapid and simple DNA extraction method for the detection of enterotoxigenic Staphylococcus aureus directly from food samples: comparison of PCR and LAMP methods. Journal of applied microbiology, 113(1), 106–113.

[172] Husakova, M., Dziedzinska, R., & Slana, I. (2017). Magnetic Separation Methods for the Detection of Mycobacterium avium subsp. paratuberculosis in Various Types of Matrices: A Review. BioMed research international, 2017, 5869854.

[173] Vingataramin, L., & Frost, E. H. (2015). A single protocol for extraction of gDNA from bacteria and yeast. BioTechniques, 58(3), 120–125.

[174] Volk, H., Piskernik, S., Kurinčič, M., Klančnik, A., Toplak, N., & Jeršek, B. (2014). Evaluation of different methods for DNA extraction from milk. Journal of Food and Nutrition Research, 53, 97–104.

[175] Pace N. R. (1997). A molecular view of microbial diversity and the biosphere. Science (New York, N.Y.), 276(5313), 734–740.

[176] Mojzsis, S. J., Arrhenius, G., McKeegan, K. D., Harrison, T. M., Nutman, A. P., & Friend, C. R. (1996). Evidence for life on Earth before 3, 800 million years ago. Nature, 384(6604), 55–59.

[177] McAlister, M. B., Kulakov, L. A., O'Hanlon, J. F., Larkin, M. J., & Ogden, K. L. (2002). Survival and nutritional requirements of three bacteria isolated from ultrapure water. Journal of industrial microbiology & biotechnology, 29(2), 75–82.

[178] McFeters, G. A., Broadaway, S. C., Pyle, B. H., & Egozy, Y. (1993). Distribution of bacteria within operating laboratory water purification systems. Applied and environmental microbiology, 59(5), 1410–1415.

[179] Shen, H., Rogelj, S., & Kieft, T. L. (2006). Sensitive, real-time PCR detects low-levels of contamination by Legionella pneumophila in commercial reagents. Molecular and cellular probes, 20(3-4), 147–153.

[180] Rand, K. H., & Houck, H. (1990). Taq polymerase contains bacterial DNA of unknown origin. Molecular and cellular probes, 4(6), 445–450.

[181] Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Kaczmarski, E. B., & Fox, A. J. (2000). Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. Journal of clinical microbiology, 38(5), 1747–1752.

[182] Patel, P., Garson, J. A., Tettmar, K. I., Ancliff, S., McDonald, C., Pitt, T., Coelho, J., & Tedder, R. S. (2012). Development of an ethidium monoazide-enhanced internally controlled universal 16S rDNA real-time polymerase chain reaction assay for detection of bacterial contamination in platelet concentrates. Transfusion, 52(7), 1423–1432.

[183] Hein, I., Schneeweiss, W., Stanek, C., & Wagner, M. (2007). Ethidium monoazide and propidium monoazide for elimination of unspecific DNA background in quantitative universal real-time PCR. Journal of microbiological methods, 71(3), 336–339.

[184] Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., Turner, P., Parkhill, J., Loman, N. J., & Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC biology, 12, 87.

[185] Xuan, C., Shamonki, J. M., Chung, A., Dinome, M. L., Chung, M., Sieling, P. A., & Lee, D. J. (2014). Microbial dysbiosis is associated with human breast cancer. PloS one, 9(1), e83744.

[186] Bhatt, A. S., Freeman, S. S., Herrera, A. F., Pedamallu, C. S., Gevers, D., Duke, F., Jung, J., Michaud, M., Walker, B. J., Young, S., Earl, A. M., Kostic, A. D., Ojesina, A. I., Hasserjian, R., Ballen, K. K., Chen, Y. B., Hobbs, G., Antin, J. H., Soiffer, R. J., Baden, L. R., … Meyerson, M. (2013). Sequence-based discovery of Bradyrhizobium enterica in cord colitis syndrome. The New England journal of medicine, 369(6), 517–528.

[187] Nadkarni, M. A., Martin, F. E., Jacques, N. A., & Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading, England), 148(Pt 1), 257–266.

[188] Horz, H. P., Vianna, M. E., Gomes, B. P., & Conrads, G. (2005). Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. Journal of clinical microbiology, 43(10), 5332–5337.

[189] Chiodini, R. J., Dowd, S. E., Davis, B., Galandiuk, S., Chamberlin, W. M., Kuenstner, J. T., McCallum, R. W., & Zhang, J. (2013). Crohn's disease may be differentiated into 2 distinct biotypes based on the detection of bacterial genomic sequences and virulence genes within submucosal tissues. Journal of clinical gastroenterology, 47(7), 612–620.

[190] Garson, J. A., Patel, P., McDonald, C., Ball, J., Rosenberg, G., Tettmar, K. I., Brailsford, S. R., Pitt, T., & Tedder, R. S. (2014). Evaluation of an ethidium monoazide-enhanced 16S rDNA real-time polymerase chain reaction assay for bacterial screening of platelet concentrates and comparison with automated culture. Transfusion, 54(3 Pt 2), 870–878.

[191] Raes, J., Korbel, J. O., Lercher, M. J., von Mering, C., & Bork, P. (2007). Prediction of effective genome size in metagenomic samples. Genome biology, 8(1), R10.

[192] Glassing, A., Dowd, S. E., Galandiuk, S., Davis, B., Jorden, J. R., & Chiodini, R. J. (2015). Changes in 16s RNA Gene Microbial Community Profiling by Concentration of Prokaryotic DNA. Journal of microbiological methods, 119, 239–242.

[193] Willerslev, E., Hansen, A. J., & Poinar, H. N. (2004). Isolation of nucleic acids and cultures from fossil ice and permafrost. Trends in ecology & evolution, 19(3), 141–147.

[194] Kearney, M. F., Spindler, J., Wiegand, A., Shao, W., Anderson, E. M., Maldarelli, F., ... & Coffin, J. M. (2012). Multiple sources of contamination in samples from patients reported to have XMRV infection. PloS one, 7(2), e30889.

[195] Cooper, A., & Poinar, H. N. (2000). Ancient DNA: do it right or not at all. Science (New York, N.Y.), 289(5482), 1139.

[196] McInnes, P., & Cutting, M. (2010). Human Microbiome Project—Core microbiome sampling protocol A. National Institutes of Health Human Microbiome Project Initiative, 12.

[197] World Health Organization. WHO/HTM/2016.13. Geneva: World Health Organization; 2016. Global Tuberculosis Report 2016. www.who.int/TB/laboratory/mycobacteriology- 444 laboratory-manual.pdf.

[198] Prasad R., Gupta N., Banka A. (2017), 2025 too short time to eliminate tuberculosis from India. Lung India, 34(5), 409-410.

[199] Ministry of Health and Family Welfare, Government of India. INDIA TB REPORT 2022. Available from: https://tbcindia.gov.in/WriteReadData/IndiaTBReport2022/TBAnnaulReport 2022.pdf, accessed on February 20, 2023.

[200] Rajpal, S., & Arora, V. K. (2020). Latent TB (LTBI) treatment: challenges in India with an eye on 2025: "To Treat LTBI or not to treat, that is the question". Indian Journal of Tuberculosis, 67(4), S43-S47.

[201] Sharma, N., Basu, S., & Chopra, K. K. (2019). Achieving TB elimination in India: The role of latent TB management. The Indian journal of tuberculosis, 66(1), 30–33.

[202] World Health Organization. The END TB strategy; 2015. Available from: https://apps.who.int/iris/bitstream/handle/10665/331326/WHO-HTM-TB-2015.19-eng.pdf?sequence=1&isAllowed=y, accessed on February 20, 2023.

[203] Central TB Division, Directorate General of Health Services, Ministry of Health and Famly Welfare, Government of India. National strategic plan for tuberculosis elimination 2017-2025. Available from: https://tbcindia.gov.in/WriteReadData/NSP Draft 20.02.2017 1.pdf, accessed on February 20, 2023.

[204] Reid, M. J. A., Arinaminpathy, N., Bloom, A., Bloom, B. R., Boehme, C., Chaisson, R., Chin, D. P., Churchyard, G., Cox, H., Ditiu, L., Dybul, M., Farrar, J., Fauci, A. S., Fekadu, E., Fujiwara, P. I., Hallett, T. B., Hanson, C. L., Harrington, M., Herbert, N., Hopewell, P. C., ... Goosby, E. P. (2019). Building a tuberculosis-free world: The Lancet Commission on tuberculosis. Lancet (London, England), 393(10178), 1331–1384.

[205] Mondal R., Jain A. (2007), XDR Mycobacterium tuberculosis isolates, India (letter) (Last assessed on 2017 May 22) Emerg Infect Dis. 2007, 13, 1429–14231.

[206] Thomas A., Ramachandran R., Rehaman F., Jaggarajamma K., Santha T., Selvakumar N., Krishnan N., Sunder MN., Sundaram V., Wares F., Narayanan, P R. (2007), Management of multi drug resistance tuberculosis in the field: Tuberculosis Research Centre experience. Indian J Tuberc., 54, 117–24.

[207] Ryu YJ. (2015), Diagnosis of pulmonary tuberculosis: recent advances and diagnostic algorithms. Tuberc Respir Dis (Seoul)., 78(2), 64-71.

[208] Yadav, J., Verma, S., Chaudhary, D., Jaiwal, P. K., & Jaiwal, R. (2019). Tuberculosis: Current Status, Diagnosis, Treatment and Development of Novel Vaccines. Current pharmaceutical biotechnology, 20(6), 446–458.

[209] Kashyap, R. S., Nayak, A. R., Husain, A. A., Shekhawat, S. D., Satav, A. R., Jain, R. K., Raje, D. V., Daginawala, H. F., & Taori, G. M. (2016). Impact of socioeconomic status and living condition on latent tuberculosis diagnosis among the tribal population of Melghat: A cohort study. Lung India : official organ of Indian Chest Society, 33(4), 372–380.

[210] Pai, M., Bhaumik, S., & Bhuyan, S. S. (2017). India's plan to eliminate tuberculosis by 2025: converting rhetoric into reality. BMJ global health, 2(2), e000326.

[211] World Health Organization. (2016). Global Tuberculosis Report 2016. Geneva: World Health Organization; 2016.

[212] Padayatchi, N., Daftary, A., Naidu, N., Naidoo, K., & Pai, M. (2019). Tuberculosis: treatment failure, or failure to treat? Lessons from India and South Africa. BMJ global health, 4(1), e001097.

[213] Pai, M., Nicol, M. P., & Boehme, C. C. (2016). Tuberculosis Diagnostics: State of the Art and Future Directions. Microbiology spectrum, 4(5), 10.1128/microbiolspec.TBTB2-0019-2016.

[214] Dash M. (2013). Drug resistant tuberculosis: a diagnostic challenge. Journal of postgraduate medicine, 59(3), 196–202.

[215] Thakur, H. (2008). Drug resistance in tuberculosis control: a global and Indian situation. Journal of Preventative Medicine (Romania), 16(3-4), 3-9.

[216] Jittimanee, S. X., Madigan, E. A., Jittimanee, S., & Nontasood, C. (2007). Treatment default among urban tuberculosis patients, Thailand. International journal of nursing practice, 13(6), 354–362.

[217] Sharma, N., Khanna, A., Chandra, S., Basu, S., Chopra, K. K., Singla, N., Babbar, N., & Kohli, C. (2020). Trends & treatment outcomes of multidrug-resistant tuberculosis in Delhi, India (2009-2014): A retrospective record-based study. The Indian journal of medical research, 151(6), 598–603.

[218] Sandhu G. K. (2011). Tuberculosis: current situation, challenges and overview of its control programs in India. Journal of global infectious diseases, 3(2), 143–150.

[219] Rawal, T., & Butani, S. (2016). Combating Tuberculosis Infection: A Forbidding Challenge. Indian journal of pharmaceutical sciences, 78(1), 8–16.

[220] Pai, M., & Furin, J. (2017). Tuberculosis innovations mean little if they cannot save lives. eLife, 6, e25956.

[221] Khaparde, S. (2019). The national strategic plan for tuberculosis step toward ending tuberculosis by 2025. Journal of Mahatma Gandhi Institute of Medical Sciences, 24(1), 17.

[222] Smithwick RW., Stratigos CB., David HL. (1975), Use of cetylpyridinium chloride and sodium chlo-ride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of Mycobacterium tuberculosis. J Clin Microbiol., 1, 411-3.

[223] Paramasivan CN., Narayana SL., Prabhaker, Rajgopal MS., Somasundaram PR., Tripathy SP. (1983), Effect of storage of sputum specimen at room temperature on smears and culture results. Tubercle., 64, 119-124.

[224] Bobadilla-del-Valle M., Ponce-de-León A., Kato-Maeda M., Hernández-Cruz A., Calva- Mercado JJ., Chávez-Mazari B., Caballero-Rivera BA., Nolasco-García JC., Sifuentes-Osornio J. (2003), Comparison of sodium carbonate, cetylpyridinium chloride, and sodium borate for preservation of sputa for culture of Mycobacterium tuberculosis. J Clin Microbiol., 41(9), 4487-4488.

[225] Ubags, N. D. J., & Marsland, B. J. (2017). Mechanistic insight into the function of the microbiome in lung diseases. The European respiratory journal, 50(3), 1602467.

[226] Peleg, A. Y., Choo, J. M., Langan, K. M., Edgeworth, D., Keating, D., Wilson, J., Rogers, G. B., & Kotsimbos, T. (2018). Antibiotic exposure and interpersonal variance mask the effect of ivacaftor on respiratory microbiota composition. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society, 17(1), 50–56.

[227] Purcell, P., Jary, H., Perry, A., Perry, J. D., Stewart, C. J., Nelson, A., Lanyon, C., Smith, D. L., Cummings, S. P., & De Soyza, A. (2014). Polymicrobial airway bacterial communities in adult bronchiectasis patients. BMC microbiology, 14, 130.

[228] Lucas, S. K., Yang, R., Dunitz, J. M., Boyer, H. C., & Hunter, R. C. (2018). 16S rRNA gene sequencing reveals site-specific signatures of the upper and lower airways of cystic fibrosis patients. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society, 17(2), 204–212.

[229] Tunney, M. M., Einarsson, G. G., Wei, L., Drain, M., Klem, E. R., Cardwell, C., Ennis, M., Boucher, R. C., Wolfgang, M. C., & Elborn, J. S. (2013). Lung microbiota and bacterial abundance in patients with bronchiectasis when clinically stable and during exacerbation. American journal of respiratory and critical care medicine, 187(10), 1118–1126.

[230] Denton, M. D. C., Foweraker, J., Govan, J., Hall, M., Isalska, B., & Jones, A. (2010). Laboratory standards for processing microbiological samples from people with cystic fibrosis: report of the UK cystic fibrosis trust microbiology laboratory standards working group. Kent, United Kingdom: Cystic Fibrosis Trust.

[231] Gjerde, D. T., Hoang, L., & Hornby, D. (2009). RNA purification and analysis: sample preparation, extraction, chromatography. John Wiley & Sons -VCH, Weinheim, Germany, 1st edition.

[232] Kojima, K., & Ozawa, S. (2002). Method for isolating and purifying nucleic acids. U.S. Patent No. 2002/0192667 A1, December 2002.

[233] Pontiroli, A., Travis, E. R., Sweeney, F. P., Porter, D., Gaze, W. H., Mason, S., Hibberd, V., Holden, J., Courtenay, O., & Wellington, E. M. (2011). Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. PloS one, 6(3), e17916.

[234] Xu, H., & Hebert, M. D. (2005). A novel EB-1/AIDA-1 isoform, AIDA-1c, interacts with the Cajal body protein coilin. BMC cell biology, 6(1), 23.

[235] Bull, T. J., McMinn, E. J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R., & Hermon-Taylor, J. (2003). Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. Journal of clinical microbiology, 41(7), 2915–2923.

[236] Cheng, J., Bull, T. J., Dalton, P., Cen, S., Finlayson, C., & Hermon-Taylor, J. (2005). Mycobacterium avium subspecies paratuberculosis in the inflamed gut tissues of patients with Crohn's disease in China and its potential relationship to the consumption of cow's milk: a preliminary study. World Journal of Microbiology and Biotechnology, 21, 1175-1179.

[237] Ellingson, J. L., Cheville, J. C., Brees, D., Miller, J. M., & Cheville, N. F. (2003). Absence of Mycobacterium avium subspecies paratuberculosis components from Crohn's disease intestinal biopsy tissues. Clinical medicine & research, 1(3), 217-226.

[238] Miller, J. M., Jenny, A. L., & Ellingson, J. L. (1999). Polymerase chain reaction identification of Mycobacterium avium in formalin-fixed, paraffin-embedded animal tissues. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 11(5), 436–440.

[239] Challans, J. A., Stevenson, K., Reid, H. W., & Sharp, J. M. (1994). A rapid method for the extraction and detection of Mycobacterium avium subspecies paratuberculosis from clinical specimens. The Veterinary record, 134(4), 95–96.

[240] Imirzalioglu, C., Dahmen, H., Hain, T., Billion, A., Kuenne, C., Chakraborty, T., & Domann, E. (2011). Highly specific and quick detection of Mycobacterium avium subsp. paratuberculosis in feces and gut tissue of cattle and humans by multiple real-time PCR assays. Journal of clinical microbiology, 49(5), 1843–1852.

[241] Quist, C. F., Nettles, V. F., Manning, E. J., Hall, D. G., Gaydos, J. K., Wilmers, T. J., & Lopez, R. R. (2002). Paratuberculosis in key deer (Odocoileus virginianus clavium). Journal of Wildlife Diseases, 38(4), 729-737.

[242] Del Prete, R., Quaranta, M., Lippolis, A., Giannuzzi, V., Mosca, A., Jirillo, E., & Miragliotta, G. (1998). Detection of Mycobacterium paratuberculosis in stool samples of patients with inflammatory bowel disease by IS900-based PCR and colorimetric detection of amplified DNA. Journal of microbiological methods, 33(2), 105-114.

[243] Pathak, S., Awuh, J. A., Leversen, N. A., Flo, T. H., & Åsjø, B. (2012). Counting mycobacteria in infected human cells and mouse tissue: a comparison between qPCR and CFU. PLoS One, 7(4), e34931.

[244] Takahashi, T., & Nakayama, T. (2006). Novel technique of quantitative nested real-time PCR assay for Mycobacterium tuberculosis DNA. Journal of clinical microbiology, 44(3), 1029-1039.

[245] El Khechine, A., Henry, M., Raoult, D., & Drancourt, M. (2009). Detection of Mycobacterium tuberculosis complex organisms in the stools of patients with pulmonary tuberculosis. Microbiology, 155(7), 2384-2389.

[246] Flores, E., Rodriguez, J. C., García-Pachón, E., Soto, J. L., Ruiz, M., Escribano, I., & Royo, G. (2009). Real-time PCR with internal amplification control for detecting tuberculosis: method design and validation. Apmis, 117(8), 592-597.

[247] Broccolo, F., Scarpellini, P., Locatelli, G., Zingale, A., Brambilla, A. M., Cichero, P., Sechi, L. A., Lazzarin, A., Lusso, P., & Malnati, M. S. (2003). Rapid diagnosis of mycobacterial infections and quantitation of Mycobacterium tuberculosis load by two real-time calibrated PCR assays. Journal of clinical microbiology, 41(10), 4565–4572.

[248] Buck, G. E., O'Hara, L. C., & Summersgill, J. T. (1992). Rapid, simple method for treating clinical specimens containing Mycobacterium tuberculosis to remove DNA for polymerase chain reaction. Journal of clinical microbiology, 30(5), 1331–1334.

[249] Cleary, T. J., Roudel, G., Casillas, O., & Miller, N. (2003). Rapid and specific detection of Mycobacterium tuberculosis by using the Smart Cycler instrument and a specific fluorogenic probe. Journal of clinical microbiology, 41(10), 4783–4786.

[250] Cox, H., Hargreaves, S., & Ismailov, G. (2003). Effect of multidrug resistance on global tuberculosis control. Lancet (London, England), 362(9398), 1858–1859.

[251] Desjardin, L. E., Chen, Y., Perkins, M. D., Teixeira, L., Cave, M. D., & Eisenach, K. D. (1998). Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis. Journal of clinical microbiology, 36(7), 1964–1968.

[252] Drobniewski, F., Balabanova, Y., & Coker, R. (2004). Clinical features, diagnosis, and management of multiple drug-resistant tuberculosis since 2002. Current opinion in pulmonary medicine, 10(3), 211–217.

[253] Dye, C., Scheele, S., Dolin, P., Pathania, V., & Raviglione, M. C. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA, 282(7), 677–686.

[254] Forbes, B. A., & Hicks, K. E. (1993). Direct detection of Mycobacterium tuberculosis in respiratory specimens in a clinical laboratory by polymerase chain reaction. Journal of clinical microbiology, 31(7), 1688–1694.

[255] Khan, I. U., & Yadav, J. S. (2004). Development of a single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. Journal of clinical microbiology, 42(1), 453–457.

[256] Kocagöz, T., Yilmaz, E., Ozkara, S., Kocagöz, S., Hayran, M., Sachedeva, M., & Chambers, H. F. (1993). Detection of Mycobacterium tuberculosis in sputum samples by polymerase chain reaction using a simplified procedure. Journal of clinical microbiology, 31(6), 1435–1438.

[257] Kox, L. F., Rhienthong, D., Miranda, A. M., Udomsantisuk, N., Ellis, K., van Leeuwen, J., van Heusden, S., Kuijper, S., & Kolk, A. H. (1994). A more reliable PCR for detection of Mycobacterium tuberculosis in clinical samples. Journal of clinical microbiology, 32(3), 672–678.

[258] Miller, N., Cleary, T., Kraus, G., Young, A. K., Spruill, G., & Hnatyszyn, H. J. (2002). Rapid and specific detection of Mycobacterium tuberculosis from acid-fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real-time PCR. Journal of clinical microbiology, 40(11), 4143–4147.

[259] Nolte, F. S., Metchock, B., McGowan, J. E., Jr, Edwards, A., Okwumabua, O., Thurmond, C., Mitchell, P. S., Plikaytis, B., & Shinnick, T. (1993). Direct detection of Mycobacterium tuberculosis in sputum by polymerase chain reaction and DNA hybridization. Journal of clinical microbiology, 31(7), 1777–1782.

[260] Noordhoek, G. T., Kolk, A. H., Bjune, G., Catty, D., Dale, J. W., Fine, P. E., … & Svenson, S. B. (1994). Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. Journal of clinical microbiology, 32(2), 277-284.

[261] Rantakokko-Jalava, K., & Jalava, J. (2002). Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. Journal of clinical microbiology, 40(11), 4211–4217.

[262] Ruiz, M., Torres, M. J., Llanos, A. C., Arroyo, A., Palomares, J. C., & Aznar, J. (2004). Direct detection of rifampin- and isoniazid-resistant Mycobacterium tuberculosis in auramine-rhodamine-positive sputum specimens by real-time PCR. Journal of clinical microbiology, 42(4), 1585–1589.

[263] Shrestha, N. K., Tuohy, M. J., Hall, G. S., Reischl, U., Gordon, S. M., & Procop, G. W. (2003). Detection and differentiation of Mycobacterium tuberculosis and nontuberculous mycobacterial isolates by real-time PCR. Journal of clinical microbiology, 41(11), 5121–5126.

[264] Torres, M. J., Criado, A., Ruiz, M., Llanos, A. C., Palomares, J. C., & Aznar, J. (2003). Improved real-time PCR for rapid detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis clinical isolates. Diagnostic microbiology and infectious disease, 45(3), 207–212.

[265] van Doorn, H. R., Claas, E. C., Templeton, K. E., van der Zanden, A. G., te Koppele Vije, A., de Jong, M. D., Dankert, J., & Kuijper, E. J. (2003). Detection of a point mutation associated with high-level isoniazid resistance in Mycobacterium tuberculosis by using real-time PCR technology with 3'-minor groove binder-DNA probes. Journal of clinical microbiology, 41(10), 4630–4635.

[266] Wilson, S. M., McNerney, R., Nye, P. M., Godfrey-Faussett, P. D., Stoker, N. G., & Voller, A. (1993). Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. Journal of clinical microbiology, 31(4), 776–782.

[267] Angra, P. K., Taylor, T. H., Iademarco, M. F., Metchock, B., Astles, J. R., & Ridderhof, J. C. (2012). Performance of tuberculosis drug susceptibility testing in U.S. laboratories from 1994 to 2008. Journal of clinical microbiology, 50(4), 1233–1239.

[268] Jiang, G. L., Chen, X., Song, Y., Zhao, Y., Huang, H., & Kam, K. M. (2013). First proficiency testing of second-line anti-tuberculosis drug susceptibility testing in 12 provinces of China. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 17(11), 1491–1494.

[269] Salamon, H., Yamaguchi, K. D., Cirillo, D. M., Miotto, P., Schito, M., Posey, J., Starks, A. M., Niemann, S., Alland, D., Hanna, D., Aviles, E., Perkins, M. D., & Dolinger, D. L. (2015). Integration of published information into a

resistance-associated mutation database for Mycobacterium tuberculosis. The Journal of infectious diseases, 211 Suppl 2(Suppl 2), S50–S57.

[270] Van Deun, A., Wright, A., Zignol, M., Weyer, K., & Rieder, H. L. (2011). Drug susceptibility testing proficiency in the network of supranational tuberculosis reference laboratories. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 15(1), 116–124.

[271] World Health Organization. (2018). The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (No. WHO/CDS/TB/2018.19) Geneva, Switzerland: World Health Organization; 2018b.

[272] McNerney, R., Clark, T. G., Campino, S., Rodrigues, C., Dolinger, D., Smith, L., Cabibbe, A. M., Dheda, K., & Schito, M. (2017). Removing the bottleneck in whole genome sequencing of Mycobacterium tuberculosis for rapid drug resistance analysis: a call to action. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases, 56, 130–135.

[273] Chatterjee, M., Bhattacharya, S., Karak, K., & Dastidar, S. G. (2013). Effects of different methods of decontamination for successful cultivation of Mycobacterium tuberculosis. The Indian journal of medical research, 138(4), 541–548.

[274] Metcalfe, J. Z., Streicher, E., Theron, G., Colman, R. E., Penaloza, R., Allender, C., Lemmer, D., Warren, R. M., & Engelthaler, D. M. (2017). Mycobacterium tuberculosis Subculture Results in Loss of Potentially Clinically Relevant Heteroresistance. Antimicrobial agents and chemotherapy, 61(11), e00888-17.

[275] Engström A. (2016). Fighting an old disease with modern tools: characteristics and molecular detection methods of drug-resistant Mycobacterium tuberculosis. Infectious diseases (London, England), 48(1), 1–17.

[276] Cho, W. H., Won, E. J., Choi, H. J., Kee, S. J., Shin, J. H., Ryang, D. W., & Suh, S. P. (2015). Comparison of AdvanSure TB/NTM PCR and COBAS TaqMan MTB PCR for Detection of Mycobacterium tuberculosis Complex in Routine Clinical Practice. Annals of laboratory medicine, 35(3), 356–361.

[277] Huggett, J. F., McHugh, T. D., & Zumla, A. (2003). Tuberculosis: amplification-based clinical diagnostic techniques. The international journal of biochemistry & cell biology, 35(10), 1407–1412.

[278] Denkinger, C. M., Kik, S. V., Cirillo, D. M., Casenghi, M., Shinnick, T., Weyer, K., Gilpin, C., Boehme, C. C., Schito, M., Kimerling, M., & Pai, M. (2015). Defining the needs for next generation assays for tuberculosis. The Journal of infectious diseases, 211 Suppl 2(Suppl 2), S29–S38.

[279] Natrajan, M., Sridhar, R., Narendran, G., Ramachandran, G., Kumar, V. V., & Tripathy, S. (2019). Crusade for TB: Bringing Treatment to Masses at their Doorsteps. The Indian journal of medical research, 149(Suppl), S89–S93.

[280] Haldar, S., Chakravorty, S., Bhalla, M., De Majumdar, S., & Tyagi, J. S. (2007). Simplified detection of Mycobacterium tuberculosis in sputum using smear microscopy and PCR with molecular beacons. Journal of medical microbiology, 56(Pt 10), 1356–1362.

[281] Kolk, A. H., Noordhoek, G. T., de Leeuw, O., Kuijper, S., & van Embden, J. D. (1994). Mycobacterium smegmatis strain for detection of Mycobacterium tuberculosis by PCR used as internal control for inhibition of amplification and for quantification of bacteria. Journal of clinical microbiology, 32(5), 1354–1356.

[282] Nagdev, K. J., Kashyap, R. S., Deshpande, P. S., Purohit, H. J., Taori, G. M., & Daginawala, H. F. (2010). Determination of polymerase chain reaction efficiency for diagnosis of tuberculous meningitis in Chelex-100 extracted DNA samples. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 14(8), 1032–1038.

[283] van Embden, J. D., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P., Martin, C., McAdam, R., & Shinnick, T. M. (1993). Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. Journal of clinical microbiology, 31(2), 406–409.

[284] Bifani, P. J., Mathema, B., Kurepina, N. E., & Kreiswirth, B. N. (2002). Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains. Trends in microbiology, 10(1), 45–52.

[285] Victor, T. C., de Haas, P. E., Jordaan, A. M., van der Spuy, G. D., Richardson, M., van Soolingen, D., van Helden, P. D., & Warren, R. (2004). Molecular characteristics and global spread of Mycobacterium tuberculosis with a western cape F11 genotype. Journal of clinical microbiology, 42(2), 769–772.

[286] Somerville, W., Thibert, L., Schwartzman, K., & Behr, M. A. (2005). Extraction of Mycobacterium tuberculosis DNA: a question of containment. Journal of clinical microbiology, 43(6), 2996–2997.

[287] Boyd, R., Ford, N., Padgen, P., & Cox, H. (2017). Time to treatment for rifampicin-resistant tuberculosis: systematic review and meta-analysis. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 21(11), 1173–1180.

[288] Theron, G., Zijenah, L., Chanda, D., Clowes, P., Rachow, A., Lesosky, M., Bara, W., Mungofa, S., Pai, M., Hoelscher, M., Dowdy, D., Pym, A., Mwaba, P., Mason, P., Peter, J., Dheda, K., & TB-NEAT team (2014). Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. Lancet (London, England), 383(9915), 424–435.

[289] World Health Organization. (2008). Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy statement, 27. World Health Organization, Geneva, Switzerland.

[290] World Health Organization. (2016). The use of molecular line probe assays for the detection of resistance to second-line anti-tuberculosis drugs: policy guidance (No. WHO/HTM/TB /2016.07). World Health Organization, Geneva, Switzerland.

[291] World Health Organization. (2016). The use of loop-mediated isothermal amplification (TB-LAMP) for the diagnosis of pulmonary tuberculosis: policy guidance. World Health Organization, Geneva, Switzerland.

[292] Shete, P. B., Farr, K., Strnad, L., Gray, C. M., & Cattamanchi, A. (2019). Diagnostic accuracy of TB-LAMP for pulmonary tuberculosis: a systematic review and meta-analysis. BMC infectious diseases, 19(1), 1-11.

[293] World Health Organization. (2010). WHO endorses new rapid tuberculosis test. Geneva: World Health Organization, 9.

[294] World Health Organization. (2017). Next-generation Xpert® MTB/RIF Ultra assay recommended by WHO. WHO (Internet). World Health Organization, Geneva, Switzerland.

[295] Fan, L., Zhang, Q., Cheng, L., Liu, Z., Ji, X., Cui, Z., Ju, J., & Xiao, H. (2014). Clinical diagnostic performance of the simultaneous amplification and testing methods for detection of the Mycobacterium tuberculosis complex for smear-negative or sputum-scarce pulmonary tuberculosis in China. Chinese medical journal, 127(10), 1863–1867.

[296] Yan, L., Zhang, Q., & Xiao, H. (2017). Clinical diagnostic value of simultaneous amplification and testing for the diagnosis of sputum-scarce pulmonary tuberculosis. BMC infectious diseases, 17(1), 545.

[297] Yan, L., Tang, S., Yang, Y., Shi, X., Ge, Y., Sun, W., Liu, Y., Hao, X., Gui, X., Yin, H., He, Y., & Zhang, Q. (2016a). A Large Cohort Study on the Clinical Value of Simultaneous Amplification and Testing for the Diagnosis of Pulmonary Tuberculosis. Medicine, 95(4), e2597.

[298] Yan, L., Xiao, H., & Zhang, Q. (2016b). Systematic review: Comparison of Xpert MTB/RIF, LAMP and SAT methods for the diagnosis of pulmonary tuberculosis. Tuberculosis (Edinburgh, Scotland), 96, 75–86.

[299] Yamaguchi, N., Sasada, M., Yamanaka, M., & Nasu, M. (2003). Rapid detection of respiring Escherichia coli O157:H7 in apple juice, milk, and ground beef by flow cytometry. Cytometry. Part A : the journal of the International Society for Analytical Cytology, 54(1), 27–35.

[300] Lenaerts, J., Lappin-Scott, H. M., & Porter, J. (2007). Improved fluorescent in situ hybridization method for detection of bacteria from activated sludge and river water by using DNA molecular beacons and flow cytometry. Applied and environmental microbiology, 73(6), 2020–2023.

[301] Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America, 74(12), 5463–5467.

[302] Henson, J., Tischler, G., & Ning, Z. (2012). Next-generation sequencing and large genome assemblies. Pharmacogenomics, 13(8), 901–915.

[303] Nikolayevskyy, V., Kranzer, K., Niemann, S., & Drobniewski, F. (2016). Whole genome sequencing of Mycobacterium tuberculosis for detection of recent transmission and tracing outbreaks: A systematic review. Tuberculosis (Edinburgh, Scotland), 98, 77–85.

[304] Hatherell, H. A., Colijn, C., Stagg, H. R., Jackson, C., Winter, J. R., & Abubakar, I. (2016). Interpreting whole genome sequencing for investigating tuberculosis transmission: a systematic review. BMC medicine, 14(1), 1-13.

[305] Papaventsis, D., Casali, N., Kontsevaya, I., Drobniewski, F., Cirillo, D. M., & Nikolayevskyy, V. (2017). Whole genome sequencing of Mycobacterium tuberculosis for detection of drug resistance: a systematic review. Clinical

microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 23(2), 61–68.

[306] Norden, M. A., Kurzynski, T. A., Bownds, S. E., Callister, S. M., & Schell, R. F. (1995). Rapid susceptibility testing of Mycobacterium tuberculosis (H37Ra) by flow cytometry. Journal of clinical microbiology, 33(5), 1231–1237.

[307] Bownds, S. E., Kurzynski, T. A., Norden, M. A., Dufek, J. L., & Schell, R. F. (1996). Rapid susceptibility testing for nontuberculosis mycobacteria using flow cytometry. Journal of clinical microbiology, 34(6), 1386–1390.

[308] Kirk, S. M., Schell, R. F., Moore, A. V., Callister, S. M., & Mazurek, G. H. (1998). Flow cytometric testing of susceptibilities of Mycobacterium tuberculosis isolates to ethambutol, isoniazid, and rifampin in 24 hours. Journal of clinical microbiology, 36(6), 1568–1573.

[309] Moore, A. V., Kirk, S. M., Callister, S. M., Mazurek, G. H., & Schell, R. F. (1999). Safe determination of susceptibility of Mycobacterium tuberculosis to antimycobacterial agents by flow cytometry. Journal of clinical microbiology, 37(3), 479–483.

[310] Pina-Vaz, C., Costa-de-Oliveira, S., & Rodrigues, A. G. (2005). Safe susceptibility testing of Mycobacterium tuberculosis by flow cytometry with the fluorescent nucleic acid stain SYTO 16. Journal of medical microbiology, 54(Pt 1), 77–81.

[311] Soejima, T., Iida, K., Qin, T., Taniai, H., & Yoshida, S. (2009). Discrimination of live, anti-tuberculosis agent-injured, and dead Mycobacterium tuberculosis using flow cytometry. FEMS microbiology letters, 294(1), 74–81.

[312] Goyani, K. H., Mukhopadhyaya, P. N. (2023). Sputum-Spotted Solid Matrix Designed to Release Diagnostic-Grade Mycobacterium tuberculosis DNA Demonstrate Optimal Biocontainment Property. International Journal of Science and Research (IJSR), 12 (10), 1082-1088.

[313] Goyani, K. H., Mukhopadhyaya, P. N. (2023). Estimating Limit of Detection of M. Tuberculosis DNA as an Analyte Released from a Chemically Coated Solid Matrix and Using a WHO-approved CB-NAAT Platform. Archives of Infect Diseases & Therapy, 7(3), 79-84.