Conventional and Modern Approaches for Clinical and Laboratory Diagnosis of Tuberculosis

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Abstract

The *Mycobacterium tuberculosis* complex (MTBC) is comprised of species *M. tuberculosis, M. bovis* including *M. bovis BCG* (vaccine strain)*, M. caprae, M. pinnipedii, M. africanum, M. microti, M. canettii, "M. mungi",* and *"M. orygis"* (Woods et al., Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes: approved standard, Wayne, 2011). Mycobacteria are acid-fast bacilli and unlike most other bacteria, they have lipid-rich cell walls and due to the presence of mycolic acid, their cell walls are impermeable to a variety of disinfecting and antimicrobial agents. This makes them resistant to a variety of chemical and pharmaceutical agents. MTBC can survive harsh climates, varying temperatures and can live in deceased hosts for long periods of time (e.g. mummies). Chronic granulomatous disease caused by *M. tuberculosis* has manifestations, involving primarily lungs but sometimes other organ systems as well. MTBC are 1–10 μm in length, aerobic, non-motile, and slowly growing bacteria with 18–20-h doubling time. MTBC smear morphology shows rods that are known for their serpentine cording due to cord factor trehalose 6, 6 dimycolate. Clinical diagnosis can be done by chest X-ray, mantoux test or symptom check in conjunction with risk factors. Laboratory testing includes smear microscopy, interferon-gamma release assays, culture, rapid-detection, identification, antimicrobial susceptibility testing, and genotyping.

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Whole-genome sequencing is currently becoming a new norm for direct direction, identification, antimicrobial susceptibility prediction or confirmation and outbreak/contact tracing/contamination investigations. Whole-genome sequencing results in large amounts of data and the bioinformatic tools for analyzing this data remain complex.

Keywords

Mycobacterium tuberculosis · Isoniazid · Rifampin · Antimicrobial susceptibility testing · Genotyping · Whole genome sequencing

10.1 Introduction

10.1.1 *Mycobacterium tuberculosis* and Tuberculosis

Mycobacterium tuberculosis is a pathogenic bacterium belonging to phylum actinobacteria, order actinomycetales and mycobacteriacae family. *M. tuberculosis* primarily infects the lungs and is the causative agent for tuberculosis (TB) infection but can manifest in many organ systems such as cerebrospinal fluid, etc. The bacterium can linger around in infected organs for years and decades. Infection occurs when bacteria are released from a contagious individual via coughing or other expulsions with excessive force. The infectious dose is 1–10 *M. tuberculosis* bacilli and a droplet particle generated by an infectious person can contain 1–3 or more bacterial cells (World Health Organization 2020; Forbes et al. 2018). These droplets can be inhaled by another individual and infect pulmonary macrophages of the host.

Active infection is a symptomatic form of TB disease that is culture positive for *M. tuberculosis*. A person with this type of disease may be infectious and require isolation. Active infection can develop in approximately 5–10% of infected individuals. The first infection is termed as primary TB (World Health Organization 2020; Forbes et al. 2018), which may go unnoticed in 90–95% of individuals, become latent, and remain so for a person's lifetime. Primary TB presents with hilar enlargement, unilateral parenchymal infiltrates and/or pleural fluid. The linear or alveolar densities are usually small and appear early as small calcified 'granulomatous' lesions predominantly in the lower lobes. In active TB, Ghon complex may be a pathological indicator of TB disease progression. The lesion in lungs is of a granulomatous inflammation and adjacent lymph node. Miliary TB represents unchecked haematogenous dissemination of mycobacteria resulting in foci either at the time of primary disease or later during reactivation. Reactivation of TB typically presents with infiltrates in the upper lung zones with or without cavitations or with a miliary pattern TB. Extrapulmonary TB is a disease of other human organs (not lungs) such as TB lymphadenitis, pleural TB, genitourinary TB, skeletal TB, meningeal TB or pericardial TB (Centers for Disease Control and Prevention 2016a, d; World Health Organization 2020; Forbes et al. 2018).

Latent TB infection or LTBI is an asymptomatic form of TB infection that is smear- and culture-negative for *M. tuberculosis*. In these individuals, infection remains under immune control, which is effective at limiting infection (Centers for Disease Control and Prevention 2016a, b, c). A healthy individual can harbour a LTBI for their lifetime, but in instances where the immune system becomes weakened such as immunosupression, HIV, autoimmune disease etc., the dormant bacteria can become active in 5–10% of the cases and is termed as secondary TB (World Health Organization 2020; Centers for Disease Control and Prevention 2016a, b, c).

10.1.2 Tuberculosis Epidemiology

TB is the leading cause of death, globally, from a single infectious bacterial agent. In 2019, TB caused disease in 10 million people worldwide, resulting in 1.2 million deaths from TB among HIV-negative individuals and 208,000 deaths in HIV-positive individuals (World Health Organization 2020). SARS CoV-2 infections have hindered TB diagnosis and treatment globally due to limited medical access. The majority of TB cases globally occur in Africa and Southeast Asia and the Western Pacific regions, with global percentages of 25%, 44%, and 18% respectively (Table 10.1, Fig. 10.1). In contrast, Europe and the Americas harbour only 2.5% and 2.9% of global TB cases (World Health Organization 2020). Eight countries account for 2/3rd of the global TB burden namely in descending order: India, Indonesia, China, Philippines, Pakistan, Nigeria, Bangladesh and South Africa (Table 10.1). From 2015 to 2019, a total of 78 countries are on track to reach the 2020 milestone of a 20% reduction in TB incidence. In 2019, an estimated 3.3% of new TB cases and 18% of previously treated cases had multi-drug-resistant TB (i.e. there were an estimated 465,000 incident cases of rifampicin-resistant TB); 78% had multi-drug-resistant TB. India (27%), China (14%) and the Russian Federation (8%) had the largest share of the global burden (World Health Organization 2018). Overall, drug resistance of *M. tuberculosis* in Canada occurs at relatively low rates (LaFreniere et al. 2018). 8.1% of tested isolates in 2017 were resistant to at least one first-line anti-TB drug; most were mono-resistant and no tested isolates were XDR-TB (LaFreniere et al. 2018).

10.2 Drug Resistance in Tuberculosis

The inappropriate and inadequate antibiotic use for the treatment of bacterial infections results in the development of drug-resistant bacteria, which has progressively made the treatment of infections more cumbersome (Laxminarayan et al. 2013). *M. tuberculosis,* in particular, antibiotic-resistant organisms are more difficult to treat, can increase the cost of treatment, time to recovery, and rate of patient mortality (World Health Organization 2020). While many bacterial species are able to gain resistance genes through horizontal gene transfer (HGT), *M. tuberculosis*

Table 10.1 Best estimated values for epidemiological burden of TB in 2019 for 30 high burden countries, WHO region and globally (rates per 100,000 population). © World Health Organization (2020). Global tuberculosis report 2020. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo)

Country	Total TB incidence[a]	HIV prevalence among incident TB cases (%)	HIV-negative TB mortality	HIV-positive TB mortality[b]
Angola	351	7.6	53	8.2
Bangladesh	221	0.19	24	0.093
Brazil	46	11	2.3	0.87
Cambodia	287	2.7	17	2.5
Central African Rep.	540	25	98	61
China	58	1.6	2.2	0.15
Congo	373	29	52	40
DPR Korea[c]	513	–	–	–
DR Congo	320	11	49	11
Ethiopia	140	6.5	19	2.5
India[d]	193	2.7	32	0.69
Indonesia	312	2.2	34	1.7
Kenya	267	26	37	24
Lesotho	654	62	57	168
Liberia	308	14	56	17
Mozambique	361	34	19	18
Myanmar	322	7.8	36	5.8
Namibia	486	32	57	50
Nigeria	219	11	63	14
Pakistan	263	0.90	19	0.90
Papua New Guinea	432	3.8	47	3.5
Philippines	554	1.9	25	0.75
Russian Federation	50	23	5.8	0.88
Sierra Leone	295	13	31	8.7
South Africa	615	58	38	62
Thailand	150	10	14	2.8
UR Tanzania	237	24	35	20
Vietnam	176	3.3	9.8	2.0
Zambia	333	46	33	53
Zimbabwe	199	60	11	31
High TB burden countries	**177**	**7.8**	**21**	**3.4**
Africa	226	24	35	16
The Americas	29	10	1.7	0.58
Eastern Mediterranean	114	0.97	11	0.38

(continued)

Table 10.1 (continued)

Country	Total TB incidence[a]	HIV prevalence among incident TB cases (%)	HIV-negative TB mortality	HIV-positive TB mortality[b]
Europe	26	12	2.2	0.45
South-East Asia	217	2.7	32	1.0
Western Pacific	93	2.0	4.4	0.33
Global	**130**	**8.2**	**16**	**2.7**

[a]Rate per 100,000; numbers rounded off to significant figures
[b]Deaths among HIV-positive TB cases are classified as HIV deaths
[c]TB incidence for DPR Korea not yet approved by national authorities
[d]Estimates for India are interim, pending results from the national TB prevalence survey (2020/2021)

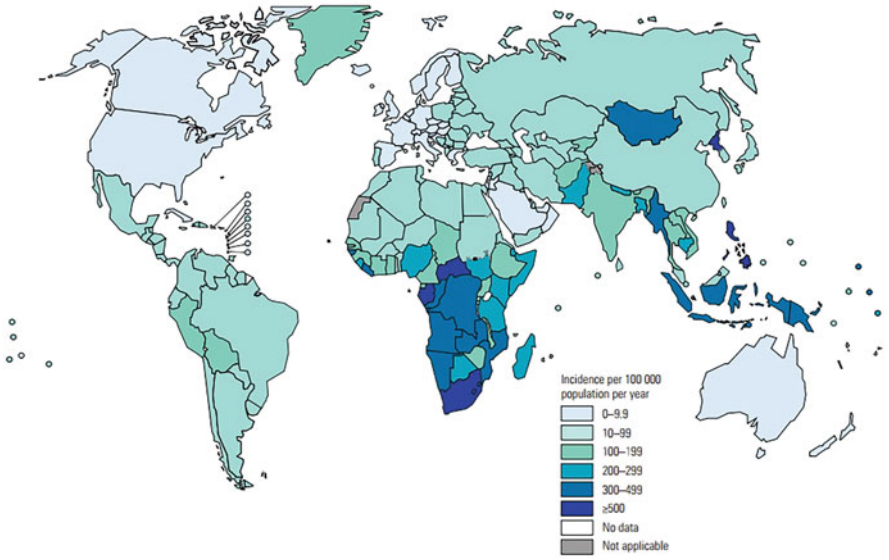

Fig. 10.1 Estimated TB incidence rates, 2019. © World Health Organization (2020). Global tuberculosis report. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO

strains gain spontaneous genomic mutations leading to resistance (Davies and Davies 2010; Schürch and Schaik 2017; Eldholm and Balloux 2016).

10.2.1 Mode of Action of Antimicrobials and Mechanisms of Drug Resistance in *M. tuberculosis*

Eldholm et al. (2014) found that *M. tuberculosis* isolates, further to evolving mutations leading to antibiotic resistance, also can independently increase fitness

over time in the presence of antibiotics. The existence of a heterogeneous population infecting an individual can further complicate treatment, as variable drug susceptibilities may be present (Nathavitharana et al. 2017). Distinct lineages of *M. tuberculosis* exist, and different mutation rates in each lineage subsequently has led to different resistances in each lineage (Ford et al. 2013). For example, strains of Lineage 2 are more likely to be resistant to multiple drugs (Ford et al. 2013). Much of the *M. tuberculosis* genomes undergo purifying selection, though antibiotics can provide pressure for positive selection of resistance mutations (Pepperell et al. 2013; Mortimer et al. 2018).

TB diagnosis and treatment is complicated due to slow growth rate of the bacteria, it's rigid and impermeable cell wall, and length and side effects of the prescribed medications. The mycolic acid content in the cell wall primarily contributes to decreased permeability of some antibiotics (Nikaido 1994). *M. tuberculosis* also has the ability to combat anti-tuberculosis drugs with various efflux systems (Balganesh et al. 2012). *M. tuberculosis* resistance is a growing problem, and has led to treatment courses that are of longer duration, more expensive and more difficult to maintain (Public Health Agency of Canada 2014). In 2016, Gallant et al. (2017) found that 9% of tested isolates were resistant to at least one drug. 83.1% of these exhibited monoresistance, this being more common than multi- or extensive-resistance.

10.2.1.1 Isoniazid

Isoniazid is a synthetic derivative of nicotinic acid with anti-mycobacterial properties. Isoniazid or INH is converted into its active form by the catalase-peroxidase enzyme encoded by the *katG* gene (Fig. 10.2) (Zhang et al. 1992). INH forms an adduct with NAD(H); competing with NAD(H) in binding to the *inhA* gene product, enoyl-acyl carrier protein reductase (Zhang et al. 1992). The gene product of *inhA* is involved in mycolic acid biosynthesis in the mycobacterial cell wall. The activated form of INH interferes with mycolic acid synthesis, making the cell wall

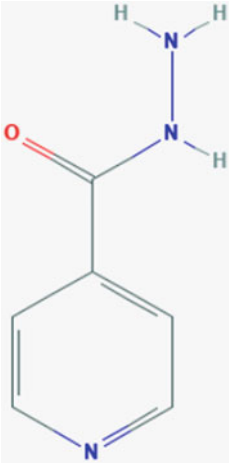

Fig. 10.2 Molecular structure of Isoniazid ($C_6H_7N_3O$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 3767, Isoniazid" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Isoniazid. Accessed 3 June 2021)

fragile (Banerjee et al. 1994). The two main causes of INH resistance include: mutations in the *katG* gene; and mutations in the *inhA* gene and promoter (Banerjee et al. 1994). Mutations in the *katG* gene lead to a decrease or to the loss of enzyme activity. Point mutations, insertions, deletions and truncations have all been identified in *katG*-mediated INH resistance (Zhang et al. 1992).

Mutations in the *inhA* promoter cause the overexpression of enoyl-acyl carrier protein reductase. Mutations in the promoter region are frequently reported in mono-resistant strains. Resistance to this drug has been associated with mutations in several genes, such as *katG, inhA, ahpC, kasA* and NDH (Palomino and Martin 2014). There is also evidence to suggest that efflux pumps and ABC transporters play a role in INH resistance (Colangeli et al. 2005; Jiang et al. 2008).

10.2.1.2 Rifampin

Rifampin or Rifampicin is a member of the class of rifamycins that is a semisynthetic antibiotic derived from *Amycolatopsis rifamycinica* (Fig. 10.3). Table 10.2 shows global incidence of rifampin resistant TB. Rifampin or RMP targets the β-subunit of RNA polymerase. The drug binds the β-subunit of the enzyme, physically blocking RNA polymerase, and thereby inhibiting RNA transcription (Blanchard 1996; Somoskovi et al. 2001, Forbes CLSI). Resistance to rifampin has been linked to mutations within the *rpoB* gene that encode the β-subunit of RNA polymerase. Within *rpoB* is an 81 bp rifampin resistance-determining region (RRDR) which is

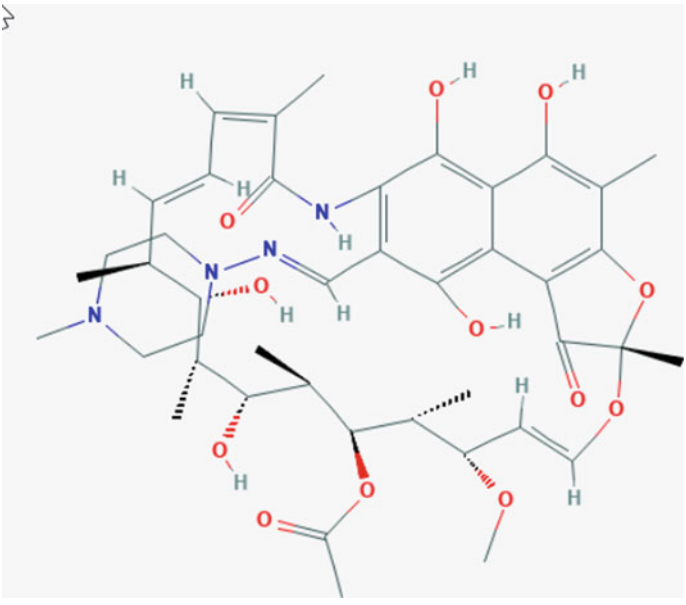

Fig. 10.3 Molecular structure of Rifampin ($C_{43}H_{58}N_4O_{12}$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 135398735, Rifampicin" PubChem, https://pubchem.ncbi.nlm.nih.gov/compound/Rifampicin. Accessed 3 June 2021)

Table 10.2 Estimated global incidence of rifampicin-resistant and/or isoniazid-resistant TB, 2019 (Number in thousands) © World Health Organization (2020). Global tuberculosis report 2020. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo)

	Rifampin resistant Best estimate	Rifampin susceptible Best estimate	Global Best estimate
Isoniazid resistant*	360	1060	1420
Isoniazid susceptible*	105	8430	8540
Global	**465**	**9490**	**9960**

*All numbers rounded to significant figures

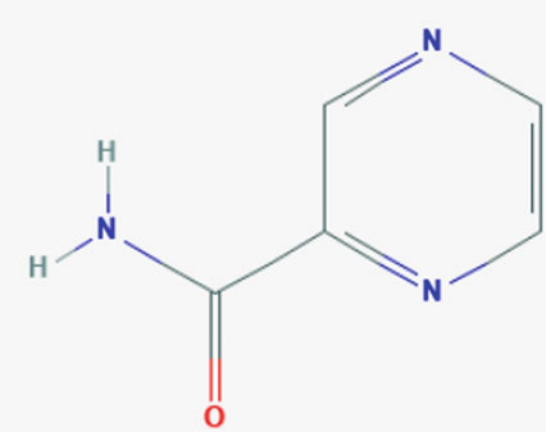

Fig. 10.4 Molecular structure of Pyrazinamide ($C_5H_5N_3O$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 1046, Pyrazinamide" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Pyrazinamide. Accessed 3 June 2021)

a mutation 'hot-spot'. The RRDR spans codons 507–533. Amino acid substitutions at codons 526 and 531 are reported to lead to high-level resistance while changes at codons 511, 516, 518, and 522 are associated with low-level resistance (Somoskovi et al. 2001). Resistance has also been associated with substitutions that occur within the *rpoB* gene, but outside the RRDR. Other mechanisms of resistance to rifampin include the permeability barrier, as well as efflux pumps, and ABC transporters (Jiang et al. 2008).

10.2.1.3 Pyrazinamide

Pyrazinamide is a synthetic pyrazinoic acid amide derivative that has bactericidal properties and is active against slowly multiplying intracellular bacilli (Fig. 10.4). Pyrazinamide or PZA is also a pro-drug that is converted into its active form of pyrazinoic acid by the enzyme nicotinamidase/pyrazinamidase (PZase) encoded by the *pncA* gene (Hazbón et al. 2006). Reported pyraziamide resistance-associated mutations are distributed along the entire length of the 561 bp *pncA* gene as well as in its promoter region. In some species of mycobacteria without *pncA* mutations, innate resistance to Pyrazinamide has been linked to a highly active pyrazinoic acid efflux mechanism (Somoskovi et al. 2001). Isolates without *pncA* mutations have also been

associated with altered pyrazinoic acid uptake and the weak binding of pyrazinoic acid to its target (Jiang et al. 2008).

10.2.1.4 Ethambutol

Ethambutol is an antibiotic with bacteriostatic, antimicrobial and antitubercular properties. Ethambutol is effective against replicating bacilli as it interferes with cell wall arabinogalactan biosynthesis (Fig. 10.5). The hypothesized target of ethambutol is the arabinosyltransferases that polymerize arabinose into arabinan (Hazbón et al. 2005). Gene transfer experiments with the *embCAB* operon, which encodes the enzymes involved in arabinogalactan biosynthesis, have provided results that suggest mutations in the *emb* operon are associated with ethambutol resistance. The most commonly occurring mutation in ethambutol-resistant *M. tuberculosis* isolates occurs within the *embB* gene (Hazbón et al. 2005). There remains about 20–35% of ethambutol-resistant isolates that do not have mutations in the *embB* gene which indicates other unknown mechanisms of ethambutol resistance do exist. An efflux pump mechanism has been associated with resistance to both ethambutol and isoniazid (Jiang et al. 2008), and occasionally resistance-conferring mutations have also been reported in *embC* (Parsons et al. 2004).

10.3 Drug Treatments

When testing samples for resistance, first-line anti-tuberculosis antimicrobials isoniazid, rifampicin, ethambutol, and pyrazinamide are the first to be tested. Second-line antimicrobials susceptibility testing can be carried out if drug resistance to first-line antimicrobials is observed (Public Health Agency of Canada 2014; Sharma et al. 2011). Public Health Agency of Canada (2014) guidelines state that the commonly used standard treatment regime for adults with fully susceptible TB disease is an initial 2-month phase of isoniazid, rifampicin, ethambutol, and potentially pyrazinamide. Ethambutol can be stopped sooner if test results determine the strain

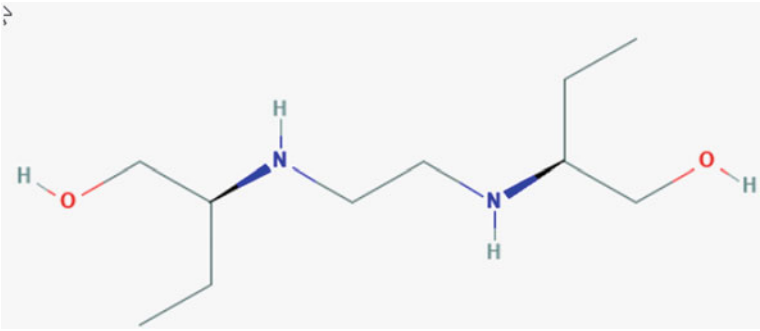

Fig. 10.5 Molecular structure of Ethambutol ($C_{10}H_{24}N_2O_2$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 14052, Ethambutol" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Ethambutol. Accessed 3 June 2021)

to be pan-sensitive. The commonly used continuation phase is administered over 4–6 months with doses of isoniazid and rifampicin. A susceptible strain is normally treated for 6–9 months and a resistant strain is normally treated for 12 to 18+ months (Gallant et al. 2017).

In Canada, resistance to isoniazid is most common (Public Health Agency of Canada 2014, 2019). In Canada, all isolates are systematically tested; strains resistant to isoniazid, or any two first-line drugs, or the multi-drug-resistant strains, are tested for second-line drugs. Treatment of drug-resistant TB often occurs with secondary antibiotics that are potentially more toxic, more expensive, and probably less effective than first-line drugs (Public Health Agency of Canada 2014). Recurrent infection can occur due to relapse with the original infection or through reinfection with a separate strain (Guerra-Assunção et al. 2014). Re-treatment cases of relapse TB are more likely to be both mono- and multi-drug resistant (MDR) than new cases (Minion et al. 2013; Dalton et al. 2012) Prior treatment with second-line drugs was a strong risk factor for resistance, and in turn increased the risk of extreme-drug resistant (XDR) TB. The second-line drugs tested included fluoroquinolones, injectables (kanamycin, amikacin, and capreomycin), and oral drugs (ethionamide and aminosalicylic acid). There are treatment guidelines in place (Falzon et al. 2011) that are attempting to reduce the accrual of drug resistance. Mixed infections occur when different strains co-infect an individual (Wang et al. 2011), and this can make diagnostics and treatment of disease more difficult (Public Health Agency of Canada 2014).

10.4 Clinical Diagnostic Tools

The primary test for clinical detection of tuberculosis is the mantoux test or tuberculin skin test, developed over 100 years ago (Davies and Pai 2008; Andersen et al. 2000). This test measures the delayed-type hypersensitivity reaction in response to TB antigens planted under the skin. A positive reaction indicates prior exposure to *M. tuberculosis*. Results are determined 48–72 h afterward by the size of swelling around the injection site (Centers for Disease Control and Prevention 2016a, b; Mayo Foundation for Medical Education and Research 2019). The sensitivity and specificity of this test is suboptimal (Andersen et al. 2000). Mantoux test shows cross-reactivity with proteins present in the Bacillus-Calmet Guerin (BCG) vaccine and with other non-tuberculous environmental *Mycobacterium* species, leading to poor test specificity (Andersen et al. 2000). The utility of this diagnostic tests is even further restricted in HIV positive and other immunocompromised patient populations as well as in children (Balcells et al. 2008; Jones et al. 1993).

Following the skin or blood test, the general next step in diagnosis is an X-ray or CT scan (Mayo Foundation for Medical Education and Research 2019). An X-ray of an individual with a TB infection can show opacities throughout the lungs, typical of pulmonary TB (U.S. National Library of Medicine 2020). While a chest X-ray cannot confirm TB diagnosis, as lesions can be indicative of other diseases, they can be used as supporting evidence of infection with laboratory results (Centers for

Disease Control and Prevention 2016a). After the clinical tests are performed, patients with suspected TB infections will usually have a sputum sample submitted to the laboratory for further testing for the presence of MTBC bacteria (Mayo Foundation for Medical Education and Research 2019).

The interferon gamma release assay is an in-vitro T-cell assay that measures the production of interferon gamma (IFN-γ) from immune cells in response to antigens specific to the RD1 (regions of deletion) region of *M. tuberculosis* that is absent from the BCG vaccine or other non-tuberculous *Mycobacterium* species (Wallis et al. 2010). White blood cells of an infected individual release interferon-γ in response to *M. tuberculosis*-derived antigens and interferon-γ release assays (IGRAs) measure this immune reactivity (Centers for Disease Control and Prevention 2016c; Mayo Foundation for Medical Education and Research 2019). The IGRA still has a limited capacity for the detection of active infection, latent case detection, false-negative test rate and restricted use in immunocompromised patients or children (Sester et al. 2011; Butera et al. 2009; Kang et al. 2005; Mahomed et al. 2006; Mori 2009). Blood IGRAs can be used in place of the Mantoux test and the test can be partly performed in hospital and a laboratory (Centers for Disease Control and Prevention 2016b).

10.5 Laboratory Diagnosis

10.5.1 Acid-Fast Bacilli Smears

Laboratory diagnosis of TB has conventionally been achieved through acid-fast bacilli (AFB) smears and culturing. AFB detection is a microscopic method which indicates the presence of acid-fast bacilli. A numbering system (1–4) is in place to quantify the bacilli seen when reporting AFB smear results. AFB smearing can utilize one of two methods: light/bright field microscopy with the traditional Ziehl-Neelsen stain, or fluorescent microscopy with an auramine stain (Christianson et al. 2013a). The Ziehl-Neelsen method utilizes a carbolfuchin stain with acid alcohol decolorizing step followed by counterstain with methylene blue counterstain. Auramine dyes (auramine-O or auramine-rhodamine) allow acid-fast bacteria to fluoresce under a fluorescent microscope (Bayot et al. 2020). Advantages of AFB smear tests are its low costs, ease of use and rapid results but disadvantages are variable, low specificity and sensitivity, sample composition, method used and subjective reporting (Public Health Agency of Canada 2014; Christianson et al. 2013a; Centers for Disease Control and Prevention 2016d; Babafemi et al. 2017; Bayot et al. 2020). A negative AFB may not necessarily rule out TB as this result could still coincide with $<$10,000 bacilli per mL of sputum (Centers for Disease Control and Prevention 2016d), as the limit of detection of AFB smears is ~10,000 organisms per mL (Lebrun et al. 1997).

10.5.2 Culture

Culturing *M. tuberculosis* is the gold standard for detecting active TB infection. It is considered the most sensitive of the conventional testing methods but is slow as results typically take 2–8 weeks (Public Health Agency of Canada 2014; Christianson et al. 2013a; Forbes et al. 2018) Culturing provides 500× the sensitivity of AFB smears and provides the added benefit of utilizing culture for further testing (Zwolska 2005). While *M. tuberculosis* can be cultured in liquid or solid media, liquid cultures have the advantage of being more rapid and sensitive, though the disadvantage of being more likely to become cross-contaminated (Cruciani et al. 2004). Culturing on solid media can take multiple weeks (Babafemi et al. 2017) for detection of growth, though some commercial broth-based systems can cut this down to 4–14 days (Centers for Disease Control and Prevention 2016d). Bactec960 MGIT, Myco-ESP culture system II, and BacT/ALERT are all automated liquid systems which are approved by Health Canada and use fluorometric or colorimetric techniques to detect culture growth. A further advantage of culture diagnosis is that this method can be performed on all specimen types (Public Health Agency of Canada 2014).

10.5.3 Drug Susceptibility Testing

Drug resistance in *M. tuberculosis* can be assayed either phenotypically or by molecular assays. Molecular methods include line-probe assays and the Xpert MTB/RIF test, along with other nucleic acid amplification methods (Public Health Agency of Canada 2014, 2019). Phenotypic assays are generally performed by incubating the infective agent with an antimicrobial on media and determining susceptibility/resistance. If an organism is unable to grow in the presence of a certain drug, it is determined to be susceptible and the drug would likely be effectively used for treatment (American Association for Clinical Chemistry 2019). Phenotypic methods can be performed on solid media which are laborious and timely, or in broth culture which are rapid and standard practice in North America (Woods et al. 2011; World Health Organization 2018). Drug susceptibility testing (DST) is normally performed for *M. tuberculosis* isolates against isoniazid, rifampicin, ethambutol, and pyrazinamide; the first-line anti-TB drugs (Centers for Disease Control and Prevention 2016d). Further DST does occur (i.e., repeat testing or second-line anti-TB drug testing) in cases in which it is warranted. Organisms may be deemed MDR if resistant to isoniazid and rifampicin. XDR-TB is determined when the organisms are resistant to isoniazid and rifampicin plus a fluoroquinolone and at least one of amikacin, kanamycin, or capreomycin (Centers for Disease Control and Prevention 2016d). DST alone cannot detect all drug resistances, prior knowledge of the mutations leading to the resistance is needed. Detection of mutations and knowledge of their significance as low-confidence or high-confidence and a quality-controlled database are extremely important. Molecular DST is normally done paired with

slow, growth-based assays as well (Sharma et al. 2011; Centers for Disease Control and Prevention 2016d).

10.5.4 Molecular Diagnostic Methods

Rapid molecular tests can be based on many principles including analysis of lipids, probe hybridization, PCR, and rRNA sequencing (Katoch 2004). Various methods of nucleic acid amplification, including PCR-based methods, can detect both the presence of MTBC and potentially drug resistance. These methods are faster than culturing methods. Commercial nucleic acid amplification assays may have high sensitivity, though are variable depending on the type of sample tested (Sarmiento et al. 2003; Ling et al. 2008a). While the sensitivity varies, the specificity of these commercial tests is ≥90% (Greco et al. 2006; Ling et al. 2008a). Commercial assays approved by Health Canada include COBAS Taqman MTB (real-time-PCR), BD ProbeTec (strand displacement amplification), Amplified MTB Direct (transcription-mediated amplification), GenoType Mycobacteria Direct (PCR) and Xpert MTB/RIF (automated cartridge-based nested PCR).

10.5.5 Xpert MTB/RIF Assay

The Xpert MTB/RIF is a cartridge-based, automated, nested, real-time PCR assay, which detects MTBC and rifampicin resistance in under 2 h (Lawn and Nicol 2011). This nucleic acid amplification-based assay mixes a sputum sample with a reagent, and the automated GeneXpert machine processes the mixture (Rachow et al. 2011). This assay is 98% specific, 85% sensitive, and rapid (Rachow et al. 2011; Steingart et al. 2013; Li et al. 2017). Advantages of this system include the very short amount of required hands-on work and the limited user infection risk due to inactivation via reagents used (Banada et al. 2010). Another benefit is that this test can be used on direct sputum samples. As opposed to AFB smearing, Xpert MTB/RIF assays have the ability to accurately differentiate between non-tuberculous mycobacteria (NTM) and MTBC (Steingart et al. 2013). The Xpert MTB/RIF assay does, however, detect both live and dead bacteria (Miotto et al. 2012). The Public Health Agency of Canada (2019) recommends that Xpert results should still be confirmed with culturing to rule out the possibility of a falsely-positive rifampicin-resistant result. Helb et al. (2009) found that the limit of detection for *M. tuberculosis* with Xpert was 4.5 genomes per reaction when DNA was used, and 131 cfu/mL when spiked sputum was tested. These authors (Helb et al. 2009) found that 23 common rifampicin resistance mutations could be detected with this assay and that after correcting for a 'conventional susceptibility test error', all susceptible samples were determined as

such. Armand et al. (2011) also found that the Xpert assay had better sensitivity for respiratory samples.

10.5.6 Line Probe Assays

Line probe assays (LPAs), such as the GenoType MTBDRplus LPA assay, are another molecular method of DST, developed for use with smear-positive sputum samples and culture isolates (Public Health Agency of Canada 2014). LPAs have a limit of detection of 10,000 cfu/mL (Ninan et al. 2016). This is a disadvantage when compared to Xpert MTB/RIF, as fewer samples may be detected with LPAs. The GenoType MTBDRplus LPA assay has been shown to have high sensitivity and specificity (>98%), though isoniazid resistance/sensitivity was inconsistent (Ling et al. 2008b). The World Health Organization has supported the use of LPAs for detection of resistance to rifampicin and isoniazid from sputum samples (World Health Organization 2008) based on detecting mutations in *rpoB* (rifampicin), *katG* (isoniazid), and *inhA* (isoniazid) genes. Brossier et al. (2010) found that the sensitivity for isoniazid resistance detection increased from 67% with the MTBDR to 86% with MTBDRplus. The GenoType MTBDR assay detects mutations only in *rpoB* and *katG*, not *inhA*, to determine resistance to rifampicin and isoniazid (Bang et al. 2006). Another variation, the GenoType MTBDR*sl*, can detect resistance in *M. tuberculosis* to ethambutol, fluoroquinolone, streptomycin, amikacin, kanamycin, and capreomycin. Fluoroquinolone resistance is detected by mutations in *gyrA* and *gyrB*, streptomycin resistance in *rpsL*, aminoglycoside/cyclic peptide resistance in *rrs* and *tlyA*, and ethambutol resistance in *embB*. This test can therefore be satisfactory for these given mutations but again, can miss mutations (which ultimately lead to resistance) in other genes or gene segments (Brossier et al. 2010).

LiPA is another LPA that can detect rifampicin resistance only, with high sensitivity (ranging 82 to 100%) and specificity (ranging 92–100%) from culture, though the sensitivity decreases with direct clinical samples. (Morgan et al. 2005).

10.5.7 PCR-Based Methods

Real-time polymerase chain reaction assay (RT-PCR), which combines PCR with fluorescent probe detection, is generally faster than conventional PCR and is comparable in sensitivity and specificity (Espy et al. 2006; Babafemi et al. 2017), but like other molecular assays, does not distinguish between viable and dead bacteria (Kralik and Ricchi 2017). While smear microscopy requires 5000–10,000 organisms per mL, RT-PCR only needs around six copies of DNA per mL (Babafemi et al. 2017). Other advantages of RT-PCR are the speed to results with accuracy and the fact that bacterial load can be quantified. Further, because this method can be automated, the required hands-on time as well as the cross-contamination risk are reduced (Katoch 2004; Sethi et al. 2012). Babafemi et al. (2017) note that this should not be used as a stand-alone assay but in support of conventional assays. Sethi et al.

(2012) found that the *mpt64* RT-PCR assay had a higher sensitivity than IS*6110* PCR. Zakham et al. (2012) found that PCR using IS*6110* had sensitivity and specificity of 92% and 98%, respectively. Copy numbers and insertion positions are variable between different strains (Thorne et al. 2011), making this an adequate marker for phylogenetic analysis and epidemiology. Choi et al. (2015) used the 16S rRNA sequence as a PCR target, differing from the commonly used IS*6110* sequence. Results of this study suggest that the 16S sequence is comparable as a PCR target to IS*6100* for *M. tuberculosis* detection with high sensitivity. 16S rRNA PCR can be a useful tool but quality databases are essential (Böttger 1989).

Warren et al. (2004) and Wang et al. (2011) developed PCR methods to detect mixed *M. tuberculosis* infections, with high sensitivity and specificity. Warren et al. (2004) noted that mixed infections were more common in cases of re-treatment. Woods et al. (2011) remind that resistance caused by mutations that are not detected by these molecular methods due to their design may still occur and as such, these tests are not perfect; interpretation of results needs to consider this fact. On the positive side, these molecular methods greatly reduce the time to results compared to culturing (i.e., 1 month versus 1 day) (Woods et al. 2011). Rapid drug-resistance results allow an earlier start of effective therapy, which leads to better outcomes for patients, overall public health, and epidemiology.

10.5.8 Genotyping

Genotyping of *M. tuberculosis* from culture is a slow and laborious process. While restriction fragment length polymorphism or spoligotyping or mycobacterial interspersed repetitive units may be methods of TB genotyping, the application of whole-genome sequencing (WGS) allows for *M. tuberculosis* identification, drug resistance prediction, in-depth investigation of strains and their genetic relatedness by use of single assay (Gardy et al. 2011; Walker et al. 2013; Cowan and Crawford 2002; Tyler et al. 2016, 2017; Pankhurst et al. 2016; Christianson et al. 2013a, b; Cowan et al. 2012; Sharma 2011). Sequence data generated from WGS can discriminate between closely related outbreaks that descend from a historical common/recent ancestor and provide inference for the direction of transmission within outbreaks. In addition, the integration of WGS with epidemiological information can identify transmission events and the presence of super-spreaders. Pankhurst et al. (Pankhurst et al. 2016) identified outbreaks that were missed by conventional methods, and demonstrated that the use of WGS for surveillance and outbreak investigations can better identify the chain of TB transmission networks. Culture-independent WGS from samples rather than cultures have the potential to expedite the reporting process by approximately 2 months. Tuberculosis is endemic in select vulnerable populations with rates of TB far exceeding the Canadian average (Patel et al. 2017; Tyler et al. 2017). The transmission of small-cluster long-term outbreaks, by use of genotyping, are being identified on an ongoing basis. Lack of infrastructure, geographic isolation, rapid tests and scarce human resources have challenged the ability of local public health officials to effectively monitor and control TB

spread (Gardy et al. 2011; Christianson et al. 2013b; Lee et al. 2015; Patel et al. 2017; Tyler et al. 2017). Newer technologies to investigate the outbreaks have emerged and evolved to help better understand the dynamics of TB transmission (Gardy et al. 2011; Walker et al. 2013; Tyler et al. 2017; Pankhurst et al. 2016). This is essential to allow public health officials to more efficiently and effectively address the transmission and spread of TB.

10.6 Whole-Genome-Sequencing-Based Advanced Diagnostics and Research

Molecular methods have advantages over conventional testing methods including the gold standard of culture, though what can be tested is limited (Bryant et al. 2015). The capability to show the gain or loss of resistance genes is not particularly relevant for TB as HGT does not occur in MTBC (Tamma et al. 2018). TB resistance generally occurs through point mutations. WGS is an appropriate method of testing for resistance as well as lineage, with the further advantage that it can be determined through a single procedure. Faster diagnosis, with accurate predictions of resistance, identification of novel mutations as well as being financially feasible are all further advantages of WGS (Walker et al. 2015; Farhat et al. 2016; Pankhurst et al. 2016; Miotto et al. 2012; Allix-Béguec et al. 2018; Zignol et al. 2018). These can lead to positive implications regarding patient networks and therapy regimes (Witney et al. 2016). WGS has some disadvantages: potential increased costs during the transition period, knowledge needed for interpretation of results, the genotype-phenotype relationship of novel markers, and presence of standards that exist for newer technologies (Rodwell 2019).

Chen et al. (2019) found that WGS could be used to predict isolate resistance to isoniazid, rifampicin, pyrazinamide, levofloxacin, amikacin, kanamycin, capreomycin, streptomycin, and prothionamide and accuracy, sensitivity, and specificity of these predictions were >85%. These authors found that the determined WGS results were overall consistent with DST results. WGS can be useful in predicting drug resistance of an isolate as well as in relating isolates for analysis of transmission and outbreaks through genotyping: the process of determining sequence variations to determine associations (Bryant et al. 2013; Roetzer et al. 2013; Walker et al. 2013; Witney et al. 2016; Integrated DNA Technologies 2020). Witney et al. (2016) note that appropriate software and bioinformatic tools are required for the ideal use of WGS methods. The costs of performing WGS versus routine DST are comparable, and so after preliminary validation, implementation is possible without increased routine costs (Witney et al. 2016).

10.6.1 Illumina and Oxford Nanopore MinION

Illumina sequencing occurs through sequencing by synthesis. Fluorescently labelled dNTPs are incorporated into DNA fragments in a massively parallel fashion.

Illumina sequencing includes four steps: library preparation by fragmentation and adapter ligation, cluster generation with adapters binding a flow cell, sequencing, and finally data analysis and alignment to a reference genome (Illumina 2017).

Recently, Oxford Nanopore sequencing has been piquing interest. Deamer et al. (2016) document the history of the development of nanopore sequencing. Oxford Nanopore's MinION sequencer, released in 2014 (Lu et al. 2016) utilizes nanopore technology to sequence DNA. Nanopore sequencing is fast, sensitive, and produces long read length WGS (Jain et al. 2015). Nanopore MinION sequencing occurs as a DNA strand is threaded through a protein pore with applied electrical current. The current flowing through the pore changes depending on which base is passing through it at any given time. This continuous change in current is used to determine the DNA sequence of the strand (Schürch and Schaik 2017; Tamma et al. 2018). This sequencing platform has an error rate between 5 and 20% (Kono and Arakawa 2019), though this issue can be accounted for by error correction and assembly strategies (Goodwin et al. 2015; Jain et al. 2015; Loman et al. 2015; Leggett et al. 2015; Schürch and Schaik 2017).

The initial investment for MinION is approximately Canadian $1000 for a basic package which includes two flow cells and a starter kit of reagents. An additional kit costs $650 for 12 samples for PCR and barcoding kit or $599 for $1D^2$ sequencing kit (R9.5). A large capital investment is required for sequencing platforms which can range from $50,000 to $100,000 for desktop sequencers, and 10× more for high-throughput platforms. MiSeq materials cost including culture and labour for sequencing is approximately $200/sample; depending on urgent (3 samples) or non-urgent submission (12 samples), and fresh or stocked culture. The type of extraction kit used will also alter the cost (Tyler et al. 2017; Brown et al. 2015; Tyler et al. 2016; Pankhurst et al. 2016).

MinION also has the advantage of real-time data analysis; the sequencing data can be analyzed as it is being produced (Judge et al. 2015; Schürch and Schaik 2017; Cao et al. 2016; Tamma et al. 2018). This allows strain identification, drug resistance detection and genotype in very little time. For slow-growing organisms like *M. tuberculosis,* this is a huge advantage (Schürch and Schaik 2017). Pankhurst et al. (2016) showed that DST prediction via WGS was sufficiently accurate (93%) while also being faster and more cost-effective than culturing methods. Illumina sequencing platforms produce reads which may not be long enough to cover various repeat elements in a bacterial genome while MinION, on the other hand, is a long-read platform, allowing more complete and greater quality bacterial genome assembly (Lu et al. 2016; Schürch and Schaik 2017). The production of long reads by nanopore sequencing helps to assemble a genome, specifically through areas with repetition and 'structural variations' potentially including indels, duplications, or inversions (Tamma et al. 2018). Bainomugisa et al. (2018) were able to assemble an isolate genome with 99.92% accuracy using only MinION reads. When Illumina reads were used to complement this data, accuracy was 99.98%. This assembly used 238x coverage and the estimated error rate of MinION data was 5.3%. Furthermore, this platform is portable due to its small size, is relatively affordable, and can quickly produce data in real-time (Lu et al. 2016; Schmidt et al. 2016). The small size and

portability of the MinION sequencer give this platform good potential for use outside of clinical laboratories (Schürch and Schaik 2017).

This platform also has some downfalls. SNPs in DNA, which can have greater effects on the protein level, may be difficult to distinguish with MinION technologies (Tamma et al. 2018). While random error should generally be removed during assembly, any systematic errors may not be as well accounted for, such as homopolymers or methylation (methylation can affect the electrical signal utilized for nanopore sequencing, which can lead to errors) (Tamma et al. 2018). The use of a method for WGS depends on urgency or the type of result needed.

10.6.2 WGS-Based Diagnostic Methods Directly from Sputum Samples

Previous studies have resulted in poor outcomes when performing WGS directly from respiratory samples but improvements by targeted enrichment via use of oligonucleotide beads to capture *M. tuberculosis* DNA prior to WGS has resulted in >20× coverage and >98% mapped genome in 83% of the study isolates (Brown et al. 2015). Developing a culture-independent diagnostic test that circumvents the lengthy 2–8-week culture step will undoubtedly lead to significant improvements in turnaround time for the *M. tuberculosis* laboratory program. However, limitations for direct WGS from sputa samples include, low bacterial load to host DNA ratio, potential *M. tuberculosis* infections, and other background microbiota that add additional challenges which will need to be addressed through both wet- and dry-laboratory strategies.

Votintseva et al. (2017) developed a method for *M. tuberculosis* DNA extraction directly from respiratory samples, a method which does not include a sample enrichment step. This allows the potential for the time to results to be less than a day when using platforms such as MinION. Votintseva et al. (2017) tested their extraction protocol with three sequencers: Illumina MiSeq, Illumina MiniSeq, and Oxford Nanopore MinION. A depth of >3× was needed for resistance predictions. 96% of the predictions made agreed with the results of DST, those that did not were a patient with a mixed infection with different resistance patterns. Brown et al. (2015) used biotinylated RNA bait to gather *M. tuberculosis* DNA from direct sputum samples. Being able to gather DNA directly from sputum allows the advantage of reduced time to diagnosis. The RNA oligo baits spanned the sequence of the entire *M. tuberculosis* genome. Once captured, DNA was then amplified and sequenced. The resulting reads had adequate depth and accurately predicted mutations associated with drug resistance (Witney et al. 2016). This method was also able to predict the presence of a mixed infection. Of the smear-positive samples, >98% of the *M. tuberculosis* genome was able to be recovered with this method in 83% of samples. For most samples, the genome coverage and depth were comparable to matched cultures. In the cases with lower coverages, it was hypothesized that these results were due to low pathogen load. The resistance predictions, compared to culture vs sputum, were in agreement. Doyle et al. (2018) evaluated WGS from

sputum samples in which mixed infections were present, noting that MGIT and solid culture do not identify these cases well (Martin et al. 2010; Hanekom et al. 2013). The authors were able to detect SNPs consistent with mixed infections in patient samples.

The challenges that are faced when trying to sequence DNA directly from sputum samples are; (a) the low amount of actual desired organisms and (b) these samples also contain DNA from cells of host, NTM and other microbes (Iketleng et al. 2018). Methods to enrich for target DNA during extraction steps can be utilized to optimize WGS results from sputum, such as Votintseva et al. (2017). The presence of different DNA sources also ultimately leads to a reduced sequencing depth of the desired genome (Doughty et al. 2014). This is particularly an issue for drug resistance detection. The advantage of real-time sequencing with MinION is that sequencing can continue until the desired coverage is reached, hence saves time when DNA load is higher and allowing continuation when it is lower. Votintseva et al. (2017) found that no false-positive resistance predictions were made, though authors did note that in order for all mutations in their catalogue to be identified, deep coverage was required. They also found systematic SNP error biases: an A to G error bias, though this could be corrected for in mapping. Even with higher error rates, deep coverage could lead to accurate resistance profiles. While rapid molecular tests can provide some information on drug resistance, WGS can provide a complete genetic profile. WGS can be a slower process but can be sped up when DNA is sequenced from sputum samples directly versus from cultured isolates. The issue with these direct samples is that the DNA can often be of poor quality and in low concentrations. This can be somewhat improved upon when a step to deplete undesired (e.g., human) DNA is included in the process. Doughty et al. (2014) used shotgun metagenomic sequencing on sputum samples with an Illumina MiSeq platform to detect *M. tuberculosis*. Their method, lacking any culturing, capturing, or amplification steps, did not provide sufficient coverage to determine drug resistance.

The large amounts of data that can be produced by WGS can lead to challenges being faced. These include methods for storing and analyzing these data as well as requiring specialized staff with the skill to analyze the data (Iketleng et al. 2018). Further limitations include needing enrichment steps, capable staff and equipment, and adequate bioinformatic systems (Lee and Behr 2015). Standardization is also difficult for assays on direct sputum samples (Lee and Behr 2015).

10.6.3 Bioinformatic Tools

There are many tools that have been developed for data analysis and error correction of WGS data. These include TB Profiler, Mykrobe Predictor TB, CASTB, KvarQ, and PhyResSE (Schleusener et al. 2017). Cao et al. (2016) developed a streaming algorithm and pipeline which can process 100× more data than the MinION computer throughput. Oxford Nanopore's Metrichor has platforms including the EPI2ME platform. This cloud-based platform allows analysis of data in real-time (Metrichor 2020). Greninger et al. (2015) developed a web-based pipeline,

MetaPORE, which couples with MinION to perform real-time analysis. MetaPORE is also a visualization tool and can identify a pathogen from Nanopore data. Raw Nanopore data is base-called using a Metrichor pipeline (Metrichor 2D Basecalling v1.14 pipeline) which is then scanned for sequence reads to subsequently be aligned using BLASTn/MegaBLAST. This can process the data to a result which corresponds to an identification. The reference sequence is determined by having the best match at each refresh, and maps are generated based on alignment. It's advantage is that it is web-based and can run on a regular laptop. Ellington et al. (2017) advocate that a single database should house all resistance information to ease comparison, updating, and curation. The Mykrobe predictor tool compares De Bruijn graphs to determine species diagnosis and predict drug resistance. This tool is able to analyze read data as is produced from MinION sequencing, and can account for multiple bacteria being present in a sample (Schürch and Schaik 2017). Bradley et al. (2015) found that sensitivity and specificity of resistance of *M. tuberculosis* using the Mykrobe predictor tool was 82.6% and 98.5%, respectively. Goodwin et al. (2015) developed Nanocorr, an error correction algorithm for Nanopore, specifically. This algorithm can handle the longer read lengths of 5–50 kb with higher error rates of 5–40%. The authors used Nanopore reads supplemented with Illumina MiSeq reads to sequence a bacterial genome. It is suggested that this method is superior to MiSeq data alone as some genomic features (rRNA, transposable elements, etc.) were better represented. Jain et al. (2015) used a tool to find SNPs and maxlik estimates. Loman et al. (2015) assembled an *E. coli* genome with Nanopore data with a three-staged method in which they detected read overlaps, corrected reads, and used a probabilistic model to 'polish' the assembly. This method realized nucleotide identity of 99.5%. The online TB Profiler tool allows reporting of drug resistance from raw sequences (Coll et al. 2015). Many other in-house developed methods and tools are also in use. Quality control, verification and validation of these tools and platforms should be done before implementing in a routine clinical laboratory (Forbes et al. 2018).

10.7 Conclusion

The causative agent of TB, *M. tuberculosis* is a slowing-growing bacteria and the methods used for identification, susceptibility testing and genotyping are primarily culture-based including liquid media culturing and susceptibility testing, 16S, *hsp65*, resistance marker gene sequencing, MIRU, spoligotyping, and restriction fragment length-based polymorphism, etc. Timely identification, treatment and surveillance of *M. tuberculosis* is hindered by the inability to rapidly and accurately identify, characterize and genotype strains directly from patient samples. This has been challenged in recent years due to SARS CoV-2 infections and outbreaks. The potential to diagnose TB, predict resistance and delineate transmission networks through the use of a single test, such as WGS, has the capability to enhance TB laboratory, TB control and prevention programs. Quality assessment of generated

sequence data, testing and validation of assays and bioinformatics pipeline parameters are all needed prior to implementation.

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