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REVISED VERSION

Real-time Next-Generation Sequencing on Shell-vial Culture to contribute to diagnosis of lymphatic tuberculosis: a case report.

Running title: NGS for lymphatic tuberculosis diagnosis

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Abstract

Lymph node tuberculosis is a of limited clinical suspicion form of *Mycobacterium tuberculosis* infection. After 15 days incubation in a cellular culture and directly from the supernatant, 11 minutes of Oxford Nanopore MinION sequencing provided a preliminary result of an antibiotic-susceptible *M. tuberculosis* Indo-Oceanic lineage strain. Oxford Nanopore MinION sequencing is a promising tool for optimising the laboratory diagnosis of lymph node tuberculosis.

Key words: Tuberculosis; *Mycobacterium tuberculosis*; real-time Next-Generation Sequencing; point-of-care; lineage.

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) complex mycobacteria are responsible for tuberculosis, which remains a major cause of morbidity and mortality in human populations worldwide [1]. The annual incidence is over 10 million cases of infection and up to 1.4 million deaths, as reported by the World Health Organization in 2019 [1]. While pulmonary tuberculosis is the main clinically recognised form of the infection, lymph node tuberculosis is of limited clinical suspicion form, in alignment with the idea that tuberculosis pathogens affect the lungs tissue and subsequently the reticuloendothelial system, causing an infection of the lymphatic system [2].

The diagnosis of lymph node tuberculosis is challenging for clinical microbiology. Besides the fact that lymph node tuberculosis may be clinically ignored for long periods, notably in the case of profound lymph node disease, and that sampling of such seriously diseased lymph nodes may be hazardous, microbiological documentation remains unsatisfactory [2,3]. Routinely used GeneXpert MTB/RIF® assays (Cepheid, Sunnyvale, CA, United States) provide rapid but only limited information as they do not distinguish between dead and live mycobacteria, nor do they distinguish between species within the *M. tuberculosis* complex. Although *M. tuberculosis stricto sensu* is the main causative agent [4], *Mycobacterium bovis* [4], *M. bovis* BCG [5], *Mycobacterium canettii* [6], *Mycobacterium africanum* [7], *Mycobacterium caprae* [8] and *Mycobacterium orygis* [9] are also rarely reported in this situation [10,11]. Moreover, sensitivity of the Xpert MTB/RIF or Xpert Ultra tests on lymph node samples remains moderate, between 70 and 81.6% for a specificity of 100% [12].

Conventional culture on egg-based medium, the gold standard for isolating *M. tuberculosis*, failed in lymph nodes diagnosis in 70% of cases [13].

Here, we combined the shell-vial protocol [11] with Next-Generation Sequencing (NGS) [10,14] in order to optimise the laboratory diagnosis of lymph node tuberculosis.

Case presentation and methods

A 22-year-old man originating from India, who had been living in France for 12 years, was admitted in our unit for a persistent fever, chills and cervical lymphadenopathy. The patient weighed 73 kg and reported no weight loss. A physical examination found no further organomegalies. A medical interview reported a family history of tuberculosis diagnosed in 2011 in his mother and his uncle. A computerized tomography scan showed necrotic lymph nodes extending from the left retropharyngeal region to the left supra-ventricular region, infracentimetric non-necrotic lymph nodes in periportal and sub-diaphragmatic regions. The pathological examination of the left cervical lymph node biopsies showed giganto-cellular epithelioid granuloma with caseous necrosis. A GeneXpert MTB/RIF® ultra-assay identified a rifampin-susceptible *M. tuberculosis* complex. The lymph node sample was inoculated on both MB/BACT MB liquid medium (Biomérieux, Marcy L'Etoile, France) and on solid medium, Coletsos [15] and Lowenstein-Jensen. The liquid medium was positive after 45 days, and replicate on solid medium grew after 48 days. Solid medium cultures were positive after 70 days of incubation. In parallel, 200 µL of biopsy fragments were inoculated onto MRC5 cells (human foetal pulmonary cells, RD-Biotech, Besançon, France). Briefly, after 1-hour incubation, the tube is centrifuged, the supernatant collected and 1.5 mL of M10 is added to the jewel tube that is then incubated at 37°C under 5% CO2 for 8 weeks (Figure 1). Cells culture is checked every 3 days and a Zielh-Neelsen staining is performed. The culture medium is changed once a week. After 15 days post-inoculation, acid-fast bacilli

were observed on shell vial cultures, while the negative control remained sterile (Figure 1). Total DNA extracted from 500 µL of the shell-vial supernatant as previously described [3] and 47 µL (1 ng/µL) DNA were incorporated into an Oxford Nanopore library preparation as previously described (14), then sequenced for 2 hours on an MinION instrument (Oxford Nanopore, Oxford, UK).

Results and discussion

A first 11-minute MinION sequencing run generated 92,000 reads, including 229 *M. tuberculosis* complex reads and 224 *M. tuberculosis sensu stricto* reads (Figure 1) analysed online using Oxford Nanopore tools designed for real-time sequencing and the EP2ME software (version 2019.11.11-2920621) [16]. After two hours of sequencing, the blast of generated reads after assembling on a Canu assembler available online on Galaxy Europe (Version 1.8) yielded a *M. tuberculosis* strain FDAARGOS-756 (GenBank accession no.: CP054014). Total reads were extracted and mapped to the *M. tuberculosis* strain FDAARGOS-756 complete genome using CLC Genomics Workbench software version 7.5.0 (Qiagen). Of the 744,732 reads in total, 2,811 were mapped to the reference genome and 4,143,715 nucleotides were matched to the reference sequence, resulting in a 93.8% genome coverage with the reference genome (Figure 1). Direct analysis of the generated reads using the TB-Profiler (Version 2.8.4+galaxy1) identified an Indo-Oceanic lineage (L.1.1.3) which was susceptible to all antibiotics. In parallel, this genome was sequenced with iSeq technology (Illumina, San Diego, USA) using the Illumina Nextera XT paired-end protocol [17]. Results were consistent with those obtained using MinION sequencing (appendix 1) [17,18].

Using a shell-vial culture protocol, we reduced the delay before a positive culture of 55-78 days compared to standard culture. The Oxford nanopore

technology genome sequencing identified the strain at the species level in 11 min and give a complete genotype in 2h while it takes 17h to sequence the complete genome using Illumina technology. The "real-time" function of the Oxford nanopore technology led to an interesting time saving, the first reads can be analysed without stopping the sequencing process. The latest published works [14,17], as well as our own experience, confirm Oxford technology is competitive in terms of cost and handling time. This cost becomes competitive compared to other molecular biology methods that require several PCRs to obtain the same level of information. The obtained reads with both genomic strategies led to the same identification and antibiotic susceptibility even though reads number and coverage were lower with MinION. Integrating both MinION and the shell-vial protocol will help refine and expand the diagnosis of lymph-node tuberculosis primarily established by the GeneXpert MTB/RIF® ultra-assay [4]. This “open” approach would even make it possible to screen for any intracellularly cultured microorganisms, specifically for the search of aetiological agents of lymphadenopathy.

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Ethics

All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management. No specific clinical sampling was performed in the course of this study.

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Competing Interests

The authors have no conflicts of interest to declare.

Authors’ contributions

MM performed the experimental design, data analysis, interpretation, and writing. MF, HA and EG collected samples and performed routine analyses. ELD and BC collected clinical data and samples. SAB performed data analysis, validation, and

writing. MD contributed to critically reviewing the manuscript, data interpretation and validation. All authors declare that they have read and approved the manuscript.

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208

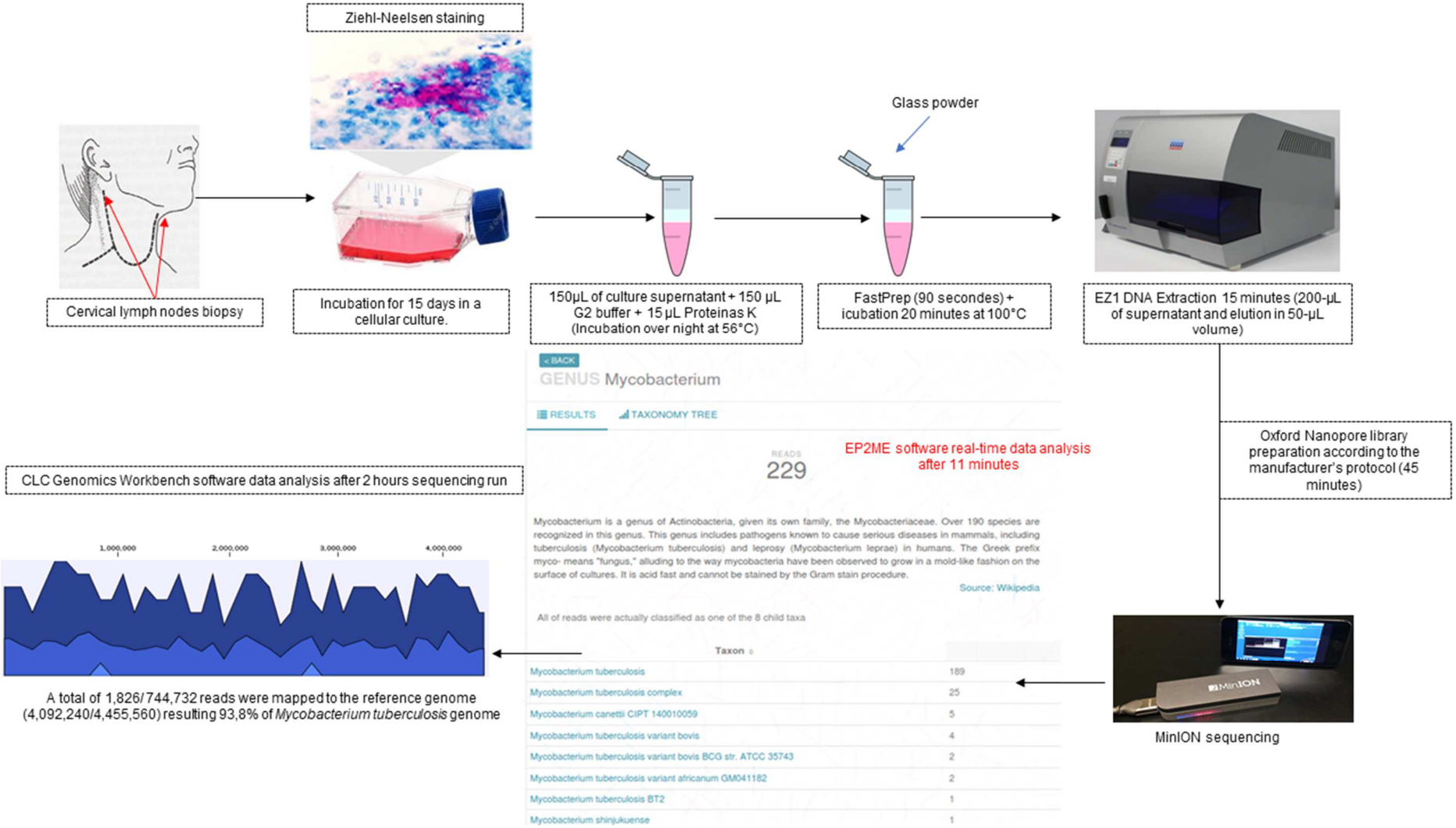
209 **Table and Figure Legends**

210 **Figure: 1**

211 **Figure 1: Two-week investigation of lymph node tuberculosis combining shell-**
212 **vial and whole genome sequencing.** A shell-vial culture inoculated with a lymph
213 node biopsy was detected positive by Ziehl-Neelsen staining after two weeks of
214 incubation. The shell-vial supernatant (500 µL) was inactivated, centrifuged for 15
215 minutes at 13,000 g and the pellet was incubated in 150 µL of G2 buffer and 15 µL of
216 proteinase K overnight at 56°C. After treatment with glass powder and incubation at
217 100°C for 10 minutes, DNA was extracted from 200 µL of supernatant using an EZ1
218 DNA kit (Qiagen) and eluted in 50 µL. A 47-µL volume of DNA was used for NGS
219 library preparation according to the Oxford Nanopore protocol. Real-time data
220 analysis was performed using EP2ME software and the *Mycobacterium tuberculosis*
221 genome was recovered by CLC software.

222

223


Ziehl-Neelsen staining
Glass powder
Cervical lymph nodes biopsy
Incubation for 15 days in a cellular culture.
150µL of culture supernatant + 150 µL G2 buffer + 15 µL Proteinas K (Incubation over night at 56°C)
FastPrep (90 secondes) + icubation 20 minutes at 100°C
EZ1 DNA Extraction 15 minutes (200-µL of supernatant and elution in 50-µL volume)
Oxford Nanopore library preparation according to the manufacturer's protocol (45 minutes)
MinION sequencing
EP2ME software real-time data analysis after 11 minutes
GENUS Mycobacterium
RESULTS
TAXONOMY TREE
229
Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. Over 190 species are recognized in this genus. This genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (Mycobacterium tuberculosis) and leprosy (Mycobacterium leprae) in humans. The Greek prefix myco- means "fungus," alluding to the way mycobacteria have been observed to grow in a mold-like fashion on the surface of cultures. It is acid fast and cannot be stained by the Gram stain procedure.
Source: Wikipedia
All of reads were actually classified as one of the 8 child taxa
Taxon
Mycobacterium tuberculosis 189
Mycobacterium tuberculosis complex 25
Mycobacterium canettii CIPT 140010059 5
Mycobacterium tuberculosis variant bovis 4
Mycobacterium tuberculosis variant bovis BCG str. ATCC 35743 2
Mycobacterium tuberculosis variant africanum GM041182 2
Mycobacterium tuberculosis BT2 1
Mycobacterium shinjukuense 1
CLC Genomics Workbench software data analysis after 2 hours sequencing run
1,000,000
2,000,000
3,000,000
4,000,000
A total of 1,826/744,732 reads were mapped to the reference genome (4,092,240/4,455,560) resulting 93,8% of Mycobacterium tuberculosis genome

224

225 **Appendix 1.** Data sequencing of the *M. tuberculosis* isolate using iSeq and MinION

	iSeq sequencing	MinION sequencing
Total reads	237510	744732
Mapped reads	209,820	2811
Average depth	20 (Max :84 ; Min :4)	3 (Max:14; Min:1)
Average read length	121.62 bp	6083,73 bp
Number of nucleotides	4233687	4143715
Genome coverage	95%	93,8%
Sequencing time	17 hours	2 hours
Lineage (TB profiler)	Indo-Oceanic (L1.1.3)	Indo-Oceanic (L1.1.3)
Antibiotic susceptibility (TB profiler)	Susceptible	Susceptible
Closest strain	*Mycobacterium tuberculosis* strain FDAARGOS_756 chromosome	*Mycobacterium tuberculosis* strain FDAARGOS_756 chromosome

226 technologies.

227