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REVISED VERSION

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

3 **Running title:** NGS for lymphatic tuberculosis diagnosis

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19 **Abstract**

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

26

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

29

30

31 **Introduction**

32 *Mycobacterium tuberculosis* (*M. tuberculosis*) complex mycobacteria are responsible
33 for tuberculosis, which remains a major cause of morbidity and mortality in human
34 populations worldwide [1]. The annual incidence is over 10 million cases of infection
35 and up to 1.4 million deaths, as reported by the World Health Organization in 2019
36 [1]. While pulmonary tuberculosis is the main clinically recognised form of the
37 infection, lymph node tuberculosis is of limited clinical suspicion form, in alignment
38 with the idea that tuberculosis pathogens affect the lungs tissue and subsequently
39 the reticuloendothelial system, causing an infection of the lymphatic system [2].

40 The diagnosis of lymph node tuberculosis is challenging for clinical microbiology.
41 Besides the fact that lymph node tuberculosis may be clinically ignored for long
42 periods, notably in the case of profound lymph node disease, and that sampling of
43 such seriously diseased lymph nodes may be hazardous, microbiological
44 documentation remains unsatisfactory [2,3]. Routinely used GeneXpert MTB/RIF®
45 assays (Cepheid, Sunnyvale, CA, United States) provide rapid but only limited
46 information as they do not distinguish between dead and live mycobacteria, nor do
47 they distinguish between species within the *M. tuberculosis* complex. Although *M.*
48 *tuberculosis stricto sensu* is the main causative agent [4], *Mycobacterium bovis* [4],
49 *M. bovis* BCG [5], *Mycobacterium canettii* [6], *Mycobacterium africanum* [7],
50 *Mycobacterium caprae* [8] and *Mycobacterium orygis* [9] are also rarely reported in
51 this situation [10,11]. Moreover, sensitivity of the Xpert MTB/RIF or Xpert Ultra tests
52 on lymph node samples remains moderate, between 70 and 81.6% for a specificity of
53 100% [12].

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

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

59 **Case presentation and methods**

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

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

86 **Results and discussion**

87 A first 11-minute MinION sequencing run generated 92,000 reads, including
88 229 *M. tuberculosis* complex reads and 224 *M. tuberculosis sensu stricto* reads
89 (Figure 1) analysed online using Oxford Nanopore tools designed for real-time
90 sequencing and the EP2ME software (version 2019.11.11-2920621) [16]. After two
91 hours of sequencing, the blast of generated reads after assembling on a Canu
92 assembler available online on Galaxy Europe (Version 1.8) yielded a *M. tuberculosis*
93 strain FDAARGOS-756 (GenBank accession no.: CP054014). Total reads were
94 extracted and mapped to the *M. tuberculosis* strain FDAARGOS-756 complete
95 genome using CLC Genomics Workbench software version 7.5.0 (Qiagen). Of the
96 744,732 reads in total, 2,811 were mapped to the reference genome and 4,143,715
97 nucleotides were matched to the reference sequence, resulting in a 93.8% genome
98 coverage with the reference genome (Figure 1). Direct analysis of the generated
99 reads using the TB-Profiler (Version 2.8.4+galaxy1) identified an Indo-Oceanic
100 lineage (L.1.1.3) which was susceptible to all antibiotics. In parallel, this genome was
101 sequenced with iSeq technology (Illumina, San Diego, USA) using the Illumina
102 Nextera XT paired-end protocol [17]. Results were consistent with those obtained
103 using MinION sequencing (appendix 1) [17,18].

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

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

121

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126 Alice Chanteloup for the cell culture.

127 **Ethics**

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

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138 **Competing Interests**

139 The authors have no conflicts of interest to declare.

140 **Authors’ contributions**

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

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

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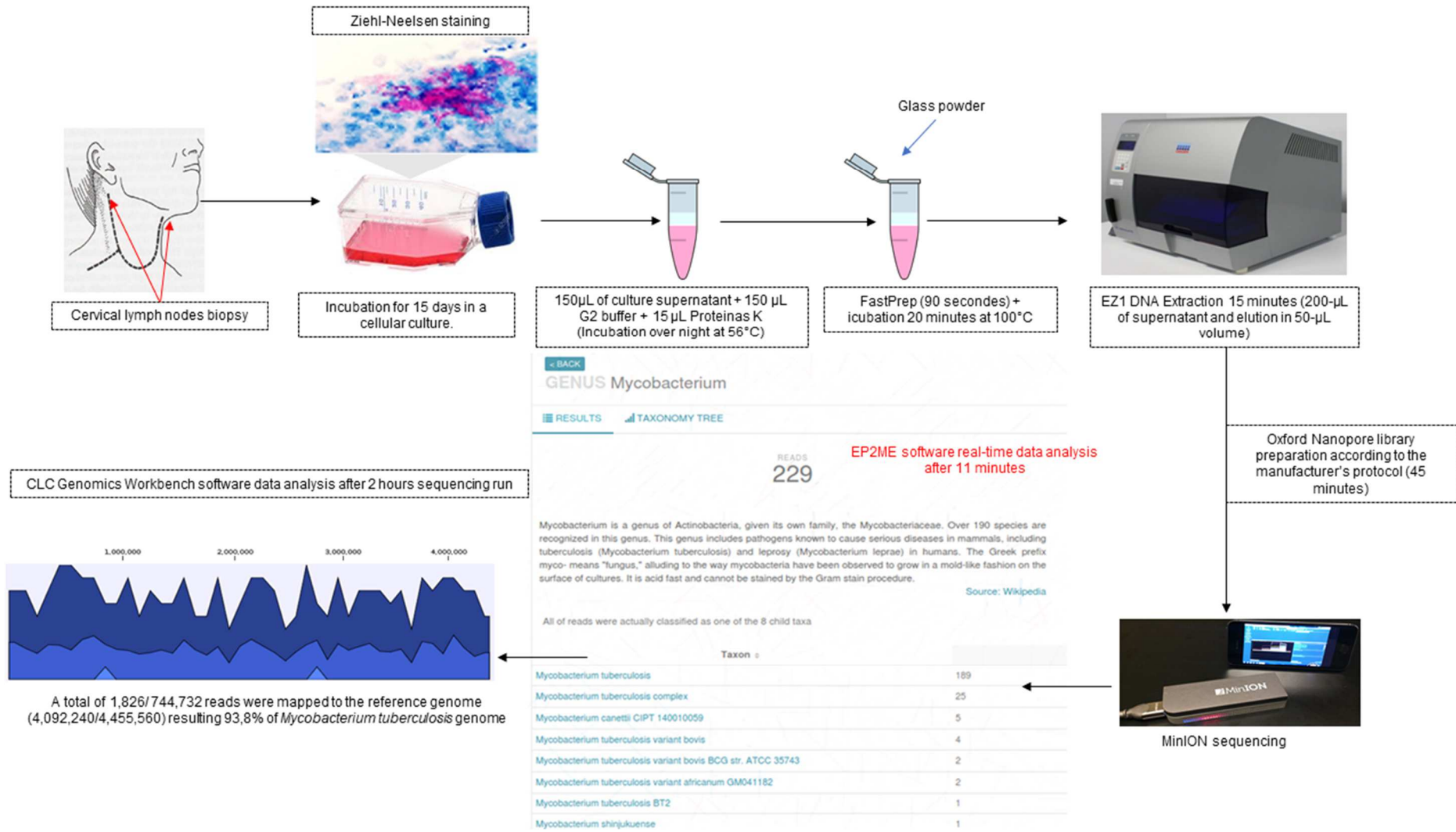
209

Table and Figure Legends210 **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



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	<i>Mycobacterium tuberculosis</i> strain FDAARGOS_756 chromosome	<i>Mycobacterium tuberculosis</i> strain FDAARGOS_756 chromosome

226 technologies.

227