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Proposition of a safe *Mycobacterium tuberculosis* complex denaturation method that does not compromise the integrity of DNA for whole-genome sequencing

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ABSTRACT

Whole-genome sequencing plays now a leading role in epidemiologic studies of tuberculosis. DNA extraction of *Mycobacterium tuberculosis* complex (MTBC) requires complete inactivation of the strains, to be handled for further molecular procedures. In this study we compared two chloroform-based denaturation methods (one with a step of heat killing, one without) to a traditional heat inactivation method. Our results showed that 40% of the strains of MTBC treated by the traditional protocol resulted in a positive culture whereas no culture was observed with the two chloroform-based protocols. The DNA extracts obtained with chloroform-based protocols preparation were successfully used for whole-genome sequencing. We recommend inactivation with our rapid and efficient denaturation method using chloroform without heat killing which met our expectations and biosecurity requirements.

1. Introduction

Manipulating *Mycobacterium tuberculosis complex* (MTBC) species from clinical specimens and cultures poses potential risks for laboratory personnel. While diagnostic samples of tuberculosis can be manipulated in biosafety level 2 (BSL2) facilities, live cultures of MTBC must be handled in a biosafety level 3 (BSL3) laboratory. Several reports of laboratory-acquired tuberculosis from aerosols or skin punctures have been reported in the literature [1–3] and highlight the need for special precautions in the manipulation of this bacteria.

Multidrug-resistant tuberculosis (MDR-TB) and extensive drug-resistant tuberculosis (XDR-TB) are major threats to global tuberculosis control. Whole-genome sequencing (WGS) plays now a leading role in epidemiologic studies of tuberculosis. It allows efficient drug resistance prediction and a better understanding of the genomic characteristics of MDR-TB [4–6]. DNA extraction is a critical step in various molecular approaches especially in WGS. Besides, it is necessary to ensure the complete inactivation of MTBC strains prior its release from a BSL3 laboratory for further manipulation.

Several studies have shown that DNA extraction procedures from MTBC cultures, including heating at 80 °C, do not ensure total microorganism inactivation and, therefore, are not completely safe [7,8]. Somerville and his colleagues demonstrated that 77,1% of the bacteria can remained alive and still subcultured on liquid media [9]. In contrast, other studies using a water bath at 80 °C during 20 min showed effective inactivation of MTBC [10,11]. These inconsistent results raised a need for careful evaluation of MTBC inactivation method. Besides, the chosen method should not compromise DNA integrity for further genomic studies. Here we evaluated a new method of inactivation on MTBC positive cultures and then we assessed DNA integrity for whole genome sequencing.

2. Material and methods

We compared the traditional and most popular method for inactivation of MTBC based on Doig's protocol [11] to a new method using organic solvent. This method can denature the mycobacterial cells and kill them without altering the DNA. To achieve this goal, we considered the very high lipid richness of the wall of mycobacteria [12]. These lipids, involved in the virulence of bacteria [13], can be extracted by the action of solvents, such as chloroform and methanol [14]. We decided to replace methanol with 70% ethanol owing to its antiseptic properties and lower toxicity. All treatments were performed under a Class 2 Microbiological Safety Cabinet in the BSL3 laboratory.

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Table 1
Number of *Mycobacterium tuberculosis* complex positive cultures obtained after treatment by the three protocols tested.

Protocol name[a]	Nb of cultures	MTBC[b]-positive cultures (%)
A	52	21 (40%)
B	52	0
C	14	0

[a] A protocol: dry water bath 80 °C, 20 min. B protocol: chloroform, 20 min; 70% ethanol, 30 min RT; dry water bath 80 °C, 15 min. C protocol: chloroform, 20 min; 70% ethanol, 30 min RT.
[b] MTBC *Mycobacterium tuberculosis* complex.

The use of chloroform requires chemical filter and all performed manipulations with this chemical agent met the biosafety requirements. The B protocol consisted of the following steps. One loopful of mycobacterial cells from Coletsos medium slants was harvested (BioRad, Marnes-la-Coquette) then dispersed into 200 µl of chloroform in a closed 1.5 ml tube and incubated for 10 min at room temperature (RT). The sample was mixed with a vortex-type stirrer and incubated for an additional 10 min. One ml of 70% ethanol was added, making sure that the reagent flows along the walls of the tube. By pipetting up and down, a suspension with a milky appearance and devoid of macroscopically visible grains was obtained. The suspension was then left at least 30 min at RT. To complete the denaturation, the tube was then incubated at 80 °C for 15 min in a dry water bath adapted to the shape of the tube. The suspension was then centrifuged at 13000 rpm for 10 min at RT. The supernatant was removed and the resulting pellet was washed with phosphate buffer saline (PBS).

B protocol described above (chloroform, 20 min RT; 70% ethanol, 30 min RT; dry water bath 80 °C, 15 min) and the traditional heat inactivation A protocol (dry water bath 80 °C, 20 min) were both evaluated on 52 strains of MTBC isolated from biological samples from different patients. As the heat inactivation step is usually the one that compromises DNA integrity leading to sequencing bias, we decided in a second time to test a third C protocol which corresponds to B protocol without the heat killing step (chloroform, 20 min RT; 70% ethanol, 30 min RT). This C protocol was evaluated on 7 strains of MTBC in duplicates (14 cultures). The pellets obtained with the three different protocols were resuspended in 1200 µl of PBS; 200 µl were inoculated on Coletsos medium and 500 µL in MGIT medium (BD, Dublin, Ireland). Both media were incubated at 37 °C for 90 and 42 days respectively and the presence or the absence of MTBC on each media was determined.

After validation of our denaturation protocol, we performed DNA extraction on the denaturated pellet of 28 strains including the *M. tuberculosis* H37Rv reference strain. Each pellet was resuspended in 500 µL of PBS and then handled outside the BSL3 laboratory and incubated overnight with 20 µL of lysozyme (100 mg/mL) (Euromedex, France). The preparation was then treated with 20 µL of proteinase K (20 mg/ml) (Magerey Nagel, Germany) 1 h at 56 °C, 5 µl of RnaseA (Macherey Nagel, Germany) 5 min at RT. DNA was then purified using modified Votintseva's protocol [6] based on AMPure XP solid phase reversible immobilization beads (Beckman Coulter, United Kingdom). DNA concentration was measured using the Qubit 2.0 fluorometer (Life Technologies, USA). Libraries were prepared using the Nextera XT DNA library Preparation kit (Illumina, USA) and paired-end sequenced on Illumina Miseq platform (Illumina, USA). Bioinformatic analyses were performed with the Phyresse web-tool (https://bioinf.fz-borstel.de/mchips/phyresse/) [15].

3. Result and discussion

Twenty one of the 52 strains treated (40%) by the traditional A protocol resulted in a positive culture in both solid and liquid media whereas no positive culture was obtained with the two chloroform-based B and C protocols ($p < 0.01$) (Table 1). The denaturation methods using chloroform being safe, DNA extraction was completed

Table 2
Results of whole-genome sequencing.

Strain inactivation			WGS results			
Name of the strain	Protocol of denaturation used[a]	DNA concentration determined by Qubit (ng/µl)	Total sequences	Depth	Coverage	Mean Phred quality score
1	B	13.5	1434219	89.35 ± 24.64	97.71%	37
2	B	0.486	993949	59.2 ± 18.43	97.61%	37
3	B	4.22	1770418	119.61 ± 43.31	97.67%	37
4	B	0.123	2007943	117.2 ± 32.74	97.18%	37
5	B	9.82	795018	49.18 ± 52.88	96.79%	37
6	B	32	674799	42.9 ± 32.97	96.7%	37
7	B	1.43	621346	42.49 ± 28.59	96.63%	37
8	B	1.75	1424985	87.79 ± 23.66	97.46%	37
9	B	4.31	700446	46.12 ± 14.33	97.86%	37
10	B	7.89	604183	40.57 ± 37.4	96.95%	37
11	B	3.86	843774	54.52 ± 17.16	97.41%	37
12	B	4.4	926406	55.5 ± 25.71	97.31%	37
13	B	23.3	929094	59.64 ± 19.33	97.7%	37
14	B	0.596	856456	59.06 ± 17.19	97.96%	37
15	B	0.2	1480623	125.88 ± 44.8	99.09%	37
15	C	8.28	817145	75.46 ± 23.28	99.01%	37
16	B	0.356	1318809	115.01 ± 42.66	99.17%	37
16	C	8.78	1433556	121.42 ± 38.39	99.09%	37
17	B	0.234	1324473	106.3 ± 44.73	98.74%	37
17	C	1.54	1462114	118.43 ± 49.39	98.72%	37
18	B	0.228	1254830	91 ± 32.93	84.95%	37
18	C	17.1	1237361	102.78 ± 34.58	96.07%	37
19	B	0.454	1087697	22.26 ± 39.29	38.67%	37
19	C	3.9	1435494	29.06 ± 49.92	38.77%	37
20	B	2.68	1360269	117.11 ± 33.78	99.34%	37
20	C	10.9	1651730	133.6 ± 36.77	99.32%	37
21	B	0.962	1340504	111.29 ± 32.83	98.72%	37
21	C	2.82	1049527	92.97 ± 28.26	99.12%	37

[a] B protocol: chloroform, 20 min RT; 70% ethanol, 30 min RT; dry water bath 80 °C, 15 min. C protocol: chloroform, 20 min RT; 70% ethanol, 30 min RT.

for 21 strains inactivated by B protocol including the *M. tuberculosis* H37Rv reference strain and 7 strains inactivated by C protocol. Concerning the 7 strains for which the two B and C protocols were performed in parallel, our results showed that the DNA integrity was better with the C protocol and the extraction yield was also better. The average DNA concentration measured was 0.73 ng/μl with the B protocol versus 7.6 ng/μl with the C protocol.

The WGS, performed on Illumina platform, has been successful for all the strains except one (Table 2). Without taking into account the sequencing results of this strain, the average of reads mapped to the H37Rv reference genome was 97.49% (84.95%–99.34%). The mean read depth was 85 × and the mean Phred Quality Score was 37 (Table 2). TB Lineage was determined in all cases and mutations associated with anti-tuberculosis drug resistance were screened. We observed high diagnostic accuracy for species identification and genetic resistance characterization was completely consistent with phenotypic drug susceptibility testing data (performed in MGIT).

These results prompted us to recommend the use of this safe preparation protocol using chloroform and ethanol without heat killing. Given its effectiveness in killing the strains, the samples could be used outside a BSL3 laboratory, while the traditional technique insufficiently ensures good safety. In agreement with the study of Somerville et al. we emphasized the need to control the initial phase of the DNA extraction, consisting of the denaturation of MTBC, in order to minimize exposure to live and possibly virulent bacteria [9]. Our results didn't confirm the ability to kill MTBC by heat, as reported by Doig et al. [11]. Our rates of regrowth by this method (40%) were in conformity with those observed by Bemer-Melchior et al. [7]. In this study, MTBC was not inactivated at 80 °C in 65% of the cultures in BACTEC vials and 52% of the culture on Lowenstein Jensen slants. In the light of these results, we recommend inactivation with our rapid and efficient denaturation method using chloroform and ethanol without heat killing, which met our expectations and biosecurity requirements. It is important to note that our chloroform–based inactivation protocol cannot be used for analysis of proteins or lipids. In addition, the protocol didn't compromise the integrity of DNA and was suitable for providing *M. tuberculosis* DNA to perform WGS on an Illumina platform. Further experimentations with our protocol should be considered before its implementation for long read sequencing using Oxford Nanopore Technologies platform.

Conflicts of interest

Authors declare no conflict of interest.

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