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Title	Direct detection of Mycobacterium bovis by a dry loop-mediated isothermal amplification assay in cattle samples collected during routine abattoir examination in Malawi
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22	Running head: Dry LAMP assay for detection of Mycobacterium bovis
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24 Abstract. The lack of quick, accurate, and low-cost detection methods has hindered the active 25 control strategies for bovine tuberculosis (bTB) in resource-limited countries with a high burden 26 of disease. We developed a dry loop-mediated isothermal amplification (LAMP) assay for rapid 27 and specific detection of Mycobacterium bovis, the principal causative agent of bTB, and 28 evaluated the efficacy of the assay using suspected bTB samples collected during routine meat 29 inspection at major regional abattoirs in Malawi. Template genomic DNA was extracted directly 30 from the granulomatous bTB-like lesion (crude extracted DNA), as well as growth from the 31 incubated mycobacterial growth indicator tubes (MGIT). Field results were visualized by the 32 naked eye within 40 min following a color change of the amplified products. The sensitivity and 33 specificity of the dry LAMP assay while using 152 DNA samples extracted from MGIT with 34 confirmed *M. bovis* results were 98% and 88%, respectively. When 43 randomly selected crude 35 DNA samples from lesions were used, the sensitivity and specificity of the dry LAMP assay 36 were 100% and 75%, respectively. Our LAMP assay offers the potential to meet the demands for 37 a low-cost and rapid field detection tool for bTB in resource-limited countries in which bTB is 38 endemic.

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40 Keywords: bovine tuberculosis; crude extracted DNA; dry LAMP assay; *Mycobacterium bovis*.

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42	Mycobacterium bovis is the causative agent of bovine tuberculosis (bTB), a chronic infectious
43	disease of mammals. ^{3,13} <i>M. bovis</i> is found mostly in cattle, in which it has caused considerable
44	economic losses as a result of milk, carcass, or organ condemnation, as well as trade
45	restrictions. ¹⁵ In addition, the zoonotic transmission of <i>M. bovis</i> to humans is a serious public
46	health hazard, particularly in places where the disease is highly prevalent in animals. ¹² In 2016,
47	the World Health Organization reported that resource-limited countries, particularly those in
48	Africa and Southeast Asia, had the highest burden of zoonotic TB. ¹⁵ Sadly, these areas are
49	challenged by either inadequate or absent bTB surveillance and control techniques. ^{1,4,9}
50	In cattle, bTB diagnosis relies on the detection of characteristic lesions during routine
51	postmortem examination, but this method is less sensitive and specific than culture and PCR. ⁸
52	On the other hand, advanced methods of detection of <i>M. bovis</i> , such as culture, PCR, and
53	sequencing, require the use of standardized laboratory equipment and technically competent
54	staff, thereby limiting their use in rural areas. ⁸ It is worth noting that the diagnosis of bTB in
55	cattle gives crucial monitoring data that can aid in the development of effective control strategies
56	to cut the transmission links between animals and humans. Therefore, there is a pressing need for
57	the development of rapid, simple, and low-cost techniques for the detection of <i>M. bovis</i> that can
58	be implemented effectively in resource-constrained areas where the bTB burden is still
59	substantial.
60	Compared with the sophisticated bTB detection approaches, the loop-mediated
61	isothermal amplification (LAMP) technique may be a better alternative for use in resource-
62	constrained areas given the simplicity, speed, specificity, and use of low-cost equipment of the

63 LAMP assay.^{5,8,11} In 2021, we reported our successful development of a conventional wet LAMP

64 method for the specific detection of *M. bovis* by targeting a 12.7-kb genomic region (region of

65 difference 4 [RD4]) in which a deletion occurs in *M. bovis* but not *M. tuberculosis* and other *M.* tuberculosis complex species.⁸ However, the wet LAMP assay still requires standardized 66 67 laboratory equipment, such as -30°C freezers to maintain a cold chain of reagents, especially 68 enzymes. Unfortunately, factors such as purchase and maintenance costs and erratic power 69 supply restrict the use of such equipment in resource-limited areas. Therefore, a dry LAMP assay 70 is a better option because the use of dried reagents eliminates the need for cold-chain storage 71 facilities. Dry LAMP assay kits can be prepared, transported, and stored easily without refrigeration.^{6,14} 72

73 We based our dry LAMP assay on our validated *M. bovis*-specific LAMP reaction system.⁸ The primers designed previously⁸ to target the sequence flanking the 12.7 kb of RD4 74 75 were used in our dry LAMP assay. This primer set included the forward inner (F1) primer 3' 76 section that bridged the RD4 deletion junction that had shown high specificity and sensitivity for *M. bovis.*⁸ A dry LAMP reaction mixture was adopted from other studies, with slight 77 modifications.^{6,14} Briefly, the dry LAMP reaction mixture was comprised of 2 µL of primer 78 79 mixture (100 µM primers: outer primers [F3 & B3], inner primers [FIP & BIP], and loop primers 80 [FLP & BLP] mixed at 1:8:6 ratio), 1.4 µL of dNTPs (25 mM); 2.5 µL of 2 M trehalose; 1 µL of 81 25× LAMP buffer (500 mM Tris-HCl [pH 8.8], 250 mM KCl); 1.8 μL of 100 mM MgSO₄; 8 U 82 Bst 2.0 WarmStart DNA polymerase (New England Biolabs); and 1 µL of colorimetric fluorescence indicator (CFI).⁸ The mixture was placed in the peripheral part of the lid of a 0.2-83 84 mL microtube and dried under a direct flow of clean air in a vacuum container (Sanplatec) 85 overnight with phosphorus oxide and silica gel. Upon visual confirmation of complete drying, 86 the kits were sealed in a light-shielding plastic bag with silica gel bags and kept at room 87 temperature.

88	The sensitivity of the dry LAMP assay was assessed by diluting <i>M. bovis</i> BCG Tokyo
89	172 DNA solution to 5 pg, 500 fg, 300 fg, 200 fg, 100 fg, and 50 fg/reaction. We performed dry
90	LAMP reactions by adding 2 μ L of diluted DNA and 23 μ L of double-distilled water (DDW) to
91	the bottom of dry LAMP assay kit tubes. The tubes were then placed in an inverted position for 2
92	min to allow the reconstitution of reagents. The reaction mixture was mixed by inverting the tube
93	5 times. ¹⁴ The controls were DDW and <i>M. tuberculosis</i> H37Rv DNA. Isothermal amplification
94	reactions were performed at 66°C for 40 min (LA-200 Loopamp real-time turbidimeter;
95	Teramecs). The detection limit was determined as 200 fg/reaction (Fig. 1A).
96	Our study was approved (protocol 19/09/2398) by the National Health Science Research
97	Committee (NHSRC), Ministry of Health and Population, Lilongwe, Malawi. We collected
98	samples during routine postmortem inspections of cattle slaughtered at 3 major regional abattoirs
99	in Malawi (Northern, Central, Southern regions) between August 2019 and February 2020. Meat
100	inspection and sample processing have been described previously. ⁷ Briefly, one tissue with a
101	bTB-like lesion was collected per animal, and the relevant demographic data were recorded. The
102	sample was transported (in a cooler box with ice packs) to the National TB Reference Laboratory
103	(NTRL; Lilongwe) for storage at -30°C until use.
104	For field evaluation, dry LAMP assay kits were transported from Japan to Malawi. We
105	performed our dry LAMP assay on DNA that was either 1) extracted directly from
106	granulomatous bTB-like lesions (crude extracted DNA) or 2) extracted from mycobacterial
107	growth indicator tubes (MGIT) cultures. Approximately 5 g of tissue was trimmed of fat and
108	ground to a paste, decontaminated by adding 4% NaOH, followed by neutralization with PBS
109	and centrifugation at 3,200 \times g for 20 min. The supernatant was discarded, and the pellet was re-
110	suspended in 1 mL of PBS. For all of the samples, 500 μ L of the re-suspended pellet was

111 inoculated in the MGIT (BACTEC 960; Becton, Dickinson) following the manufacturer's user 112 manual. For 43 randomly selected samples, the remaining 500 µL of the pellet was subjected to 113 crude DNA extraction. For both the MGIT cultures and processed pellets from granulomatous 114 bTB-like lesions, genomic DNA was extracted by 2 cycles of heating at 95°C for 15 min and 115 immediate cooling to -30°C for 30 min.⁷ Dry LAMP reactions were performed as described 116 above, and results were interpreted by observing the color change of CFI (Fig. 1B, 1C). 117 Furthermore, we performed a standard multiplex PCR (mPCR) assay that targeted the RD4, as described previously,^{2,7} to confirm the identification of isolates as *M. bovis*. Statistical analysis 118 119 was done (epiR package of R software; https://www.r-project.org/). 120 Of the 152 collected samples, 112 were positive on MGIT culture. Of these, we detected 121 85 isolates as *M. bovis* using our dry LAMP assay and confirmed by *M. bovis*-specific mPCR 122 (Table 1; Suppl. Table 1). Based on the mPCR as a reference method to detect *M. bovis*, the 123 sensitivity and specificity of our dry LAMP assay were 98% (85 positive of 87 samples tested) 124 and 88% (57 negative of 65 samples tested), respectively, compared to MGIT culture results. 125 There was excellent agreement (percentage agreement 93.4%, Cohen kappa = 0.86 [CI: 0.78– 126 0.95]) between our dry LAMP assay and the standard mPCR assay for the detection of *M. bovis* 127 (Table 1). 128 We used simple random sampling to select 43 samples from which crude DNA was 129 extracted directly from the pellets before culturing. Of these selected samples, we detected M. 130 bovis in 28 samples by our dry LAMP assay; *M. bovis* was confirmed in 23 samples by the *M.* 131 bovis-specific mPCR assay (Table 2; Suppl. Table 1). Thus, the sensitivity and specificity of our 132 dry LAMP assay with crude DNA extracted before culturing were 100% (23 of 23) and 75% (15

133 of 20), respectively (Table 2).

134 Our dry LAMP assay may prove valuable compared to other traditional methods for 135 detecting M. bovis. First, our LAMP assay identified M. bovis within a shorter time (40 min) than that taken for most PCR-based methods (typically ~3 h).^{2,10} Second, our LAMP assay procedures 136 137 are simple and do not require standardized laboratory equipment such as thermocyclers. We used 138 a previously developed battery-powered, hand-made portable LED illuminator⁶ to visualize 139 results (Fig. 1B). As well, dry LAMP assay results could be detected by the naked eye using 140 color change (Fig. 1C). Third, the use of crude DNA extracted from samples without culturing 141 removes the time taken for the mycobacteria to grow (~ 14 d) and hence expedites the 142 surveillance for bTB. Hence, targeted control strategies can be implemented quickly to control 143 the disease. Fourth, the use of dry reagents eliminates the need for cold-chain maintenance of 144 reagents given that the kits are easy to transport and can be stored at room temperature for at 145 least 6 mo. Our dry LAMP assay kits were made in Japan and transported to Malawi without any 146 requirement for a cold chain. In Malawi, we used the dry LAMP assay tubes over 3 mo. 147 One major challenge that we faced was to optimize the dry LAMP assay to use crude 148 extracted DNA. The specificity of our *M. bovis* dry LAMP assay was only 75% when DNA was 149 extracted directly from lesions (Table 2). We hypothesized that the DNA extraction method and 150 quality of DNA could have been a contributing factor. Hence, further optimization, especially of 151 the DNA extraction process, will be required. Nevertheless, our *M. bovis* dry LAMP assay 152 provides an alternative point-of-care detection method for bTB, especially in resource-limited 153 areas because of its rapidity, sensitivity, and absence of a requirement for sophisticated 154 laboratory tools.

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Declaration of conflicting interests

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164	Supplemental material
165	Supplemental material for this article is available online.
166	References
167	1. Allix-Béguec C, et al. Importance of identifying Mycobacterium bovis as a causative agent of
168	human tuberculosis. Eur Respir J 2010;35:692–694.
169	2. Bakshi CS, et al. Rapid differentiation of Mycobacterium bovis and Mycobacterium
170	tuberculosis based on a 12.7-kb fragment by a single tube multiplex-PCR. Vet Microbiol
171	2005;109:211–216.
172	3. Corner LA, et al. Mycobacterium bovis infection in the Eurasian badger (Meles meles): the
173	disease, pathogenesis, epidemiology and control. J Comp Pathol 2011;144:1-24.
174	4. Cosivi O, et al. Zoonotic tuberculosis due to Mycobacterium bovis in developing countries.
175	Emerg Infect Dis 1998;4:59–70.
176	5. Dheda K, et al. Point-of-care diagnosis of tuberculosis: past, present and future. Respirology
177	2013;18:217–232.

178	6. Hayashida K, et al. Direct blood dry LAMP: a rapid, stable, and easy diagnostic tool for
179	human African trypanosomiasis. PLoS Negl Trop Dis 2015;9:e0003578.
180	7. Kapalamula TF, et al. Molecular epidemiology of Mycobacterium bovis in central parts of
181	Malawi. Transbound Emerg Dis 2022;69:1577–1588.
182	8. Kapalamula TF, et al. Development of a loop-mediated isothermal amplification (LAMP)
183	method for specific detection of Mycobacterium bovis. PLoS Negl Trop Dis
184	2021;15:e0008996.
185	9. Müller B, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect
186	Dis 2013;19:899–908.
187	10. Nakajima C, et al. Identification of Mycobacterium tuberculosis clinical isolates in
188	Bangladesh by a species distinguishable multiplex PCR. BMC Infect Dis 2010;10:118.
189	11. Notomi T, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res
190	2000;28:E63.
191	12. Olea-Popelka F, et al. Zoonotic tuberculosis in human beings caused by Mycobacterium
192	bovis—a call for action. Lancet Infect Dis 2017;17:e21–e25.
193	13. Teppawar RN, et al. Zoonotic tuberculosis: a concern and strategies to combat. In: Enany S,
194	ed. Basic Biology and Applications of Actinobacteria. IntechOpen, 2018.
195	doi:10.5772/intechopen.76802
196	14. Thapa J, et al. Direct detection of Mycobacterium tuberculosis in clinical samples by a dry
197	methyl green loop-mediated isothermal amplification (LAMP) method. Tuberculosis
198	(Edinb) 2019;117:1–6.

- 199 15. World Health Organization, et al. Zoonotic tuberculosis. 2017.
- 200 https://cdn.who.int/media/docs/default-source/hq-tuberculosis/zoonotic-tb-factsheet-
- 201 2017.pdf?sfvrsn=66fdf3a1_3&download=true
- 202

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Table 1. Comparative analysis of Mycobacterium bovis dry LAMP assay and the

204 multiplex PCR (mPCR) based on DNA extracted from mycobacterial growth indicator tube

	mPCR							
Dry LAMP	M. bovis+	M. bovis–	Total	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Cohen kappa,* %
M. bovis+	85	8	93	98 (0.92, 1.00)	88 (0.77, 0.95)	91 (0.84, 0.96)	97 (0.88, 1.00)	86 (0.78, 0.95)
M. bovis–	2	57	59					
Total	87	65	152					

205 (MGIT) positive isolates.

206 95% CIs in parentheses. NPV = negative predictive value; PPV = positive predictive value.

207 * Determined using epiR package, p = 0.05.

208

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Table 2. Performance of Mycobacterium bovis dry LAMP assay evaluated using crude

210 extracted DNA from cattle samples, compared to multiplex PCR (mPCR) results.

	mPCR				
Dry LAMP	M. bovis+	M. bovis–	Total	Sensitivity, %	Specificity, %
M. bovis+	23	5	28	100	75
M. bovis–	0	15	15		
Total	23	20	43		

211

212

213	Figure 1. Sensitivity and observation of <i>Mycobacterium bovis</i> dry LAMP assay results.
214	A. Positive amplification of <i>M. bovis</i> DNA observed by rising curves of turbidity of amplified
215	products (LA-200 Loopamp real-time turbidimeter; Teramecs). Our dry LAMP assay detected as
216	little as 200 fg of <i>M. bovis</i> BCG Tokyo 172 genomic DNA. M.tb = <i>Mycobacterium tuberculosis</i> ;
217	NC = negative control. B. Dry LAMP assay results observed under LED light; the color change
218	is from orange to light-yellow for positive samples. C. Dry LAMP assay results observed with
219	the naked eye in natural light; the color shifts from violet to sky-blue for positive samples.
220	

221 Supplemental Table 1.

