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1 **Validation of the FluoroType® MTBDR assay for the detection of rifampicin and**
2 **isoniazid resistance in *Mycobacterium tuberculosis* complex isolates**
3
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35 **ABSTRACT**

36 For *Mycobacterial tuberculosis* complex the rapid and accurate diagnosis of drug resistance is
37 crucial to ensure early initiation of appropriate therapy. Recently a new molecular diagnostic
38 test, the FluoroType® MTBDR aimed at detecting rifampicin and isoniazid resistance has
39 become available. This study aimed to evaluate the FluoroType® MTBDR in comparison to
40 phenotypic drug susceptibility testing (DST) using *M. tuberculosis* complex isolates.
41 MTBC isolates underwent phenotypic DST and were tested using the FluoroType® MTBDR
42 and Genotype MTBDRplus. Sanger sequencing of the key regions of *rpoB, katG, inhA* and
43 *aphC* was performed for isolates with discordant phenotypic and molecular results.
44 Furthermore isolates with specific wildtype bands missing in the Genotype MTBDRplus
45 indicating the presence of a mutation were investigated by Sanger sequencing. Specificity and
46 sensitivity defined as the proportion of isolates correctly determined as susceptible and resistant
47 by the FluoroType® TBMDR compared to phenotypic DST were calculated.
48 A total of 180 culture isolates were included; phenotypic DST showed 85 isolates susceptible
49 to isoniazid and rifampicin, 7 with isoniazid mono-resistance, 7 with rifampicin mono-
50 resistance and 81 with multi-drug resistance. Specificity of the FluoroType® MTBDR was
51 100% (95%CI 96.0-100%) for both rifampicin and isoniazid. Sensitivity was 91.7% (95%CI
52 83.6-96.6%) for isoniazid and 98.9% (95%CI 93.8-100.0) for rifampicin.
53 The FluoroType® MTBDR has a high sensitivity and specificity for the detection of rifampicin
54 and isoniazid resistance when using culture isolates.

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65 **Introduction**

66 Rapid and accurate laboratory diagnosis of *Mycobacterium tuberculosis* drug susceptibility and
67 resistance are crucial to ensure early initiation of appropriate therapy, to adequately manage
68 disease and to control further transmission. Conventional drug susceptibility testing (DST)
69 relies on culture-based methods with results only available after several weeks. Much shorter
70 turn-around times can be achieved with molecular diagnostics either performed directly on
71 sputum samples or positive cultures.[1-3]

72 The currently available molecular methods include Xpert® MTB/RIF, line-probe assays
73 (LPAs), Sanger target sequencing and next generation sequencing.[4-7] LPAs are recommended
74 by the World Health Organization (WHO) as rapid diagnostic tests for detection of drug
75 resistance.[8] The technology combines PCR with subsequent reverse hybridization. The
76 specifically bound amplicons are made visible in a colorimetric detection reaction resulting in
77 banding patterns suggestive of the presence or absence of mutations in the target genes. The
78 most widely used LPA, the GenoType MTBDR*plus,* detects the most prevalent mutations in
79 the *rpoB, katG, inhA* genes conferring resistance to rifampicin (RMP) and isoniazid (INH). The
80 Genotype® MTBDR*plus* is designed to specifically detect four *rpoB* mutations (in codons 516,
81 526 and 531), two *katG* mutations (in codon 315) and four *inhA* mutations in the regulatory
82 region. Other mutations within the amplified region of the target genes are indicated by the
83 absence of a wildtype band without the simultaneous presence of a mutation band.

84 Recently a new assay, the FluoroType® MTBDR directed at the same target genes, but using a
85 different technology has been developed. This assay combines a Linear-After-The-Exponential
86 (LATE)–PCR[9] together with special probes using lights-on/lights-off detection technology.[10]
87 The readouts of the FluoroType® MTBDR are melting curves. The shapes correspond to
88 wildtypes or the presence of specific mutations. The FluoroType® MTBDR is an open system,
89 that identifies characterized mutation *via* a learning software interpreting the melting curves.
90 Practically the FluoroType® MTBDR has several advantages over the Genotype®
91 MTBDRplus i) less hands-on time ii) more rapid results ii) decreased risk of DNA
92 contamination and iv) automatic interpretation with the possibility to import results directly
93 into a laboratory information system. However, sensitivity and specificity for detecting RMP
94 and INH resistance of this new method have not been investigated under routine conditions.
95 This study aimed to compare the results of phenotypic DST, GenoType® MTBDR*plus* and
96 FluoroType® MTBDR using cultures positive for *M. tuberculosis* complex.
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99 **Methods**

100 Mycobacterial cultures were performed at the National Mycobacterial Reference Laboratory in
101 Borstel, Germany between May 2016 and October 2017 using mycobacterial growth indicator
102 tubes (MGIT) (Becton Dickinson, USA) and Loewenstein-Jensen and Stonebrink culture slants
103 (Artelt-Enclit, Germany). All cultures were identified to species level using the GenoType
104 MTBC ver 1.X (Hain Lifescience, Nehren, Germany) according to the manufacturer's
105 instructions. The MGIT 960-Isoniazid-Rifampicin-Ethambutol (IRE) kit (Becton Dickison)
106 was used according to the manufacturer's instructions. The critical concentrations of
107 Rifampicin and Isoniazid were 1 µg/ml and 0.1 µg/ml, respectively.[11]
108 For DNA-extraction an aliquot of 500 µl was obtained from the bottom of a positive MGIT or
109 two loops (10 µl) of bacterial growth were collected from Loewenstein-Jensen or Stonebrink
110 culture slants and added to 500 µl of 0.9% NaCl solution. Samples were centrifuged at 3000 x
111 g for 15 minutes. Supernatants were removed, and DNA extracted using the Fluorolyse® kit
112 (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. In brief,
113 100 µl of lysis buffer and 2 µl of internal control were added to the remaining pellets. The
114 mixed samples were incubated at 95°C for 5 minutes, centrifuged for 1 min and resuspended in
115 100 µl neutralization buffer. Following centrifugation at 10000 x g for 5 minutes supernatants
116 were transferred into a 0.5 ml cryovial. DNA was stored at -20°C. For each batch, one negative
117 control was included using 100 µl of lysis buffer, 100 µl of neutralization buffer and 2 µl of
118 internal control.
119 DNA was analysed with the Genotype MTBDR*plus* version 2 and FluoroType® MTBDR
120 assays (Hain Lifescience, Nehren, Germany) as per manufacturer's instructions. In brief, for
121 the FluoroType® MTBDR 20 µl of DNA-solution was added to 6 µl master mix component A
122 and 14 µl master mix component B and transferred into 96-well plates. Plates were then sealed,
123 centrifuged and analyzed via FluoroCycler® 96. For each plate, one positive and one negative
124 control were included. For the Genotype MTBDR*plus* version 2, 5 µl of DNA-solution were
125 added to 45 µl of master mix, containing 10µl master mix component A and 35 µl component
126 B. 20 µl of PCR-products were added to 20µl denaturation reagent in a hybridization well.
127 Hybridization reagents were prepared, preheated and loaded onto the GT-Blotter® 48 (Hain
128 Lifescience, Nehren, Germany). After an initial hybridization step, strips were added and
129 hybridization performed as per standard protocol. For each batch, one negative control (45 µl
130 master mix, 5 µl DNA free water) was tested. Interpretation of the results was performed
131 according to the manufacturer's instructions.

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132 The package insert of the FluoroType® MTBDR identifies the following *rpoB*, *katG* and *inhA*
133 mutations: T508A, S509T, E510H, L511P, S512K, Q513L, Q513P, Q513R, D516A, D516F,
134 D516V, D516Y, N518I, S522L, S522Q, H526C, H526D, H526G, H526L, H526N, H526P,
135 H526Q, H526R, H526S, H526Y, R529K, S531F, S531L, S531L, S531Q, S531W, L533E,
136 L533P, S315T1, S315T2, S315N, S315R, G-17T, A-16G, C-15T, G-9A, T-8A, T-8C and T-
137 8G. In contrast the Genotype MTBDRplus is able to detect and specify the following
138 mutations: D516V, H526Y, H526D, S531L, S315T1, S315T2, C-15T, A-16G, T-8C and T-8A.
139 Molecular results were interpreted without knowledge of phenotypic DST results. Sanger
140 sequencing in the key regions of *rpoB*, *katG, inhA and aphC*[12] was performed for isolates with
141 discordant results either between the two molecular methods or the molecular and phenotypic
142 methods. If the Genotype MTBDR*plus* showed a missing wildtype band, but not mutation band
143 Sanger sequencing in the respective key region was performed to confirm the FluoroType®
144 MTBDR result. Sanger sequencing was performed using an ABI 3130xl genetic analyzer
145 (Applied Biosystems) and an ABI BigDye Terminator cycle sequencing kit (version 3.1)
146 according to the manufacturer's instructions.
147 Molecular results were coded as wildtype, individual mutation, mutations in a region of the
148 gene and indeterminate. Interpretation of molecular results was susceptible, resistant and
149 indeterminate. Indeterminate results in the Genotype MTBDR*plus* were defined as detection of
150 MTBC DNA without the presence of a gene locus controls. All samples yielding an
151 indeterminate result were re-tested using a new aliquot of DNA. A mixed culture
152 (heteroresistance) was defined as both wild-type and mutant DNA being present either
153 identified by wild-type and mutant band (Genotype MTBDRplus) and/or respective double
154 peaks in the DNA sequence.
155 To determine the accuracy of the FluoroType® MTBDR assay, results were compared with
156 phenotypic DST results. Sensitivity and specificity were calculated for RMP and INH
157 separately. Sensitivity was defined as the proportion of isolates correctly determined as
158 resistant by the FluoroType® MTBDR compared to the phenotypic DST results. Specificity
159 was defined as the proportion of isolates correctly determined as susceptible by the
160 FluoroType® MTBDR compared to the phenotypic DST results. McNemar test on paired
161 proportions was used to compare sensitivity of the FluoroType® MTBDR and the Genotype
162 MTBDR*plus* for INH. All statistical analysis were performed using Stata 14.0.
163 Information was extracted from the laboratory information system anonymously. The ethics
164 committee of the University of Luebeck approved the study. Individual consent was not sought
165 as no additional patient information was obtained.

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167 **Results**

168 A total of 180 cultures were included in the study. The majority was identified as *M.*
169 *tuberculosis* (n=173), followed by *M. africanum* (n=5), *M. bovis ssp. bovis* (n=1) and *M. bovis*
170 *var. BCG* (n=1). Analysis of RMP and INH resistance included all isolates regardless of
171 species. 85 isolates tested phenotypically susceptible for both RMP and INH, 7 were INH
172 mono-resistant, 7 RMP mono-resistant and 81 resistant to both RMP and INH (table 1). The
173 number of indeterminate results by FluoroType® MTBDR was 1 (0.6%) for *rpoB,* 2 (1.1%) for
174 *inhA* and 6 for *katG* (3.3%) initially. Following repeated DNA extraction the number of
175 indeterminate results was 0 (0.0%) for *rpoB,* 1 (0.6%) for *inhA* and 3 for *katG* (1.7%).
176 Sensitivity of the FluoroType® MTBDR for INH was 91.7% (77/84, 95%CI 83.6-96.6) and
177 specificity 100% (92/92, 96.1-100.0). For RMP the sensitivity was 98.9% (87/88, 95%CI 93.8-
178 100.0) and specificity 100.0% (90/90, 95%CI 96.0-100.0). The most frequent *rpoB* mutation
179 detected by FluoroType® MTBDR was S531L (n=56). A total of 7 *rpoB* mutations remained
180 unidentified. Mutations were identified by Sanger *rpoB* sequencing as H526L (n=3), S531L
181 (n=1), Q513K (n=1), D516Y (n=1) and one mixed culture with a H526D and a S531L
182 mutation. A total of four mixed culture were identified in the study. One of those cultures was a
183 mixture of an *rpoB* wildtype isolate and an isolate with a L533P mutation. The FluoroType®
184 MTBDR detected the L533P mutations while the Genotype MTBDR*plus* did not. The
185 FluoroType® MTBDR identified three isolates as having H526P mutations while Sanger
186 sequencing revealed H526L mutations. The FluoroType® MTBDR falsely detected an H526R
187 mutation in a mixed culture with one isolate harboring a H526D and the other a S531L
188 mutation. Of the seven cultures falsely tested as INH susceptible by the FluoroType® MTBDR
189 four had *katG* and/or *inhA* mutations detected by the Genotype MTBDR*plus*. This resulted in a
190 higher, but non-significant sensitivity of the MTBDR*plus* (96.0%; 95%CI 89.9-99.3) for INH
191 compared to the FluoroType® MTBDR (91.7%; 95%CI 83.6-96.6) (p=0.12).

192

193 **Discussion**

194 This study shows high sensitivity and specificity of the FluoroType® MTBDR for the
195 detection of RMP and INH resistance using *M. tuberculosis* complex isolates. The proportion
196 of indeterminate results was small. Repeat DNA extraction resolved 5 of 9 indeterminate
197 results suggesting that insufficient or low quality DNA might have been the problem. Two of
198 the isolates that tested repeatedly indeterminate were mixed cultures which might explain the
199 result.

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200 For RMP, the sensitivity and specificity of FluoroType® MTBDR were comparable to those
201 reported for established molecular diagnostics such as the Genotype MTBDR*plus* and the
202 Xpert MTB/RIF.[2,3,13] While the presence or absence of an *rpoB* mutation was correctly
203 detected in all isolates, the FluoroType® MTBDR failed to identify the exact mutation in 10%
204 (9/87) of cases. Given that silent *rpoB* mutations are rare,[14-16] one might argue that detecting
205 the presence of an *rpoB* mutation itself is sufficient. In fact, the most widely used molecular
206 diagnostic test, the Xpert MTB/RIF only indicates the presence of RMP resistance without
207 information about the associated *rpoB* mutation.
208 The sensitivity of the FluoroType® MTBDR for detecting *katG* and/or *inhA* mutations was
209 lower compared to the Genotype MTBDR*plus*. Two of the isolates falsely reported as INH
210 susceptible by the FluoroType® MTBDR had a rare combination of mutations, a S315T1 *katG*
211 mutation and a C-17T mutation in the *inhA* promotor region, one isolate had a T-8A mutation
212 in the *inhA* promotor region. Theoretically the FluoroType® MTBDR has the potential for
213 continuous improvement when new mutations and their respective melting curve properties are
214 detected and fed back into the analytic software. Whether and how frequently improvement
215 through software updates will occur remains to be seen.
216 WHO recommends, that commercial molecular LPAs such as the Genotype MTBDR*plus* may
217 be used for positive sputum smear specimen or a cultured isolate of *M. tuberculosis* complex to
218 detect resistance to RMP and INH.[8] The Genotype MTBDR*plus* offers benefit compared to
219 phenotypic DST when used on cultured isolates. Firstly, results are available within 48 hours of
220 the culture becoming positive in contrast to the 7-14 days to with culture-based DST.[17]
221 Secondly, these assays are not affected by the presence of contamination with bacteria, fungi or
222 non-tuberculosis mycobacteria, as are liquid culture-based DST systems. The Genotype
223 MTBDR*plus* and FluoroType® MTBDR detect mutations in the same three target genes.
224 However, there are some distinct advantages of the FluoroType® MTBDR. Except for DNA
225 extraction, it provides a fully-automated, closed system with a capacity of testing 95 samples in
226 one batch. This in turn decreases the risk of DNA contamination, which is less problematic
227 when using DNA extracted from cultured isolates, but may be a significant problem when
228 testing primary samples. Full automation also means less hands-on time and automated
229 interpretation of results reducing the risk of analytic errors. Results can be directly imported
230 into a laboratory information system thus decreasing transcription errors.
231 This study has several strengths and limitations. It was conducted in a National Tuberculosis
232 Reference Laboratory with standardized procedures for phenotypic and molecular DST and
233 long-standing expertise with LPA and Sanger sequencing. Interpretation of molecular results

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234 was performed blinded to the phenotypic DST results. Half of the isolates included in the study
235 were resistant to both RMP and INH. However, the majority of isolates harbored the common
236 S531L *rpoB* and S315T1 *katG* mutations, possibly skewing the sensitivity estimate upwards.
237 In summary, the FluoroType® MTBDR assay testing culture isolates of *M tuberculosis*
238 complex has a high sensitivity for detecting RMP resistance, but a lesser sensitivity for
239 detection of INH resistance. It offers an alternative to the currently WHO-endorsed Genotype
240 MTBDR*plus* especially for high throughput laboratories. However, validation studies from
241 other settings are needed to ensure that isolates with a variety of mutations and combinations of
242 mutations are investigated. Furthermore studies conducted in low- and middle-income
243 countries with high burden of MDR-TB should be performed to assess the performance of the
244 new test in those settings.
245

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249 Genotype MTBDR*plus* assay kits for free. The project was partly funded by the German Centre
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313 **Table 1: Results of the FluoroType® MTBDR, GenoType MTBDR*plus* and phenotypic DST including all 180 isolates**

N	FluoroType® MTBDR					GenoType MTBDR*plus*					Phenotypic DST		Sanger Sequencing			
	rpoB	*katG*	*inhA*	Molecular DST Interpretation		*rpoB*	*katG*	*inhA*	Molecular DST Interpretation							
				RMP	**INH**				**RMP**	**INH**	**RMP**	**INH**	*rpoB*	*katG*	*inhA*	*alpC*
85[b]	WT	WT	WT	S	S	WT	WT	WT	S	S	S	S				
6	WT	S315T1	WT	S	R	WT	S315T1	WT	S	R	S	R				
1	WT	WT	C-15T	S	R	WT	WT	C-15T	S	R	S	R				
1	WT	S315T1	C-15T	S	R	WT	S315T1	C-15T	S	R	R	R	Mutation outside the hotspot region			
3	S531L	WT	WT	R	S	S531L	WT	WT	R	S	R	S				
1	S531L	WT	WT	R	S	S531L	WT	WT	R	S	R	R		WT	WT	WT
3	S531L	WT	WT	R	S	530-533	WT	WT	R	S	R	S	S531L			
1	S531L	WT	WT	R	S	530-533	WT	WT	R	S	R	R	S531L	WT	WT	C-10T
1	S531L	WT	WT	R	S	S531L	WT	T-8A	R	R	R	R			T-8A	
1	S531L	IND	WT	R	IND	S531L	IND	WT	R	IND	R	R		WT	WT	WT
1	S531L	MUT	WT	R	R	S531L	S315T1	WT	R	R	R	R		S315T1		
2	S531L	S315T2	WT	R	R	S531L	S315T2	WT	R	R	R	R				
2	S531L	S315T1	C-15T	R	R	S531L	S315T1	C-15T	R	R	R	R				
1	S531L	S315T1	C-15T	R	R	530-533	S315T1	C-15T	R	R	R	R	S531L			
1	S531L	S315T1	T-8C	R	R	S531L	S315T1	T-8C	R	R	R	R				
38	S531L	S315T1	WT	R	R	S531L	S315T1	WT	R	R	R	R				
1	S531L	S315T1	WT	R	R	530-533	S315T1	WT	R	R	R	R	S531L			
5	D516V	S315T1	WT	R	R	D516V	S315T1	WT	R	R	R	R				
1	D516V	S315T1	WT	R	R	513-519	S315T1	WT	R	R	R	R	D516V			
1[a]	D516Y	WT	IND	R	IND	513-519 S531L	IND	C-15T	R	R	R	R	D516Y S531L	WT	C-15T	
1[c]	D516Y	WT	WT	R	S	513-519	S315T1	-15 to -17	R	R	R	R	D516Y	S315T1	C-17T	

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3[c]	H526D	S315T1	WT	R	R	H526D	S315T1	WT	R	R	R	R				
1	H526D	WT	C-15T	R	R	H526D	WT	C-15T	R	R	R	R				
1	H526D	WT	WT	R	S	H526D	WT	WT	R	S	R	S				
1	H526N	S315T1	WT	R	R	526-529	S315T1	WT	R	R	R	R	H526N			
3	H526P	S315T1	C-15T	R	R	526-529	S315T1	C-15T	R	R	R	R	H526L			
1[a]	H526R	IND	WT	R	IND	H526D S531L	S315T1	WT	R	R	R	R	H526D S531L	S315T1		
1	H526Y	IND	WT	R	IND	H526Y	S315T1	WT	R	R	R	R		S315T1		
2[c]	H526Y	S315T1	WT	R	R	H526Y	S315T1	WT	R	R	R	R				
1	H526Y	WT	WT	R	S	H526Y	S315T1	WT	R	R	R	R		S315T1		
1	L533P	S315T1	T-8C	R	R	530-533	S315T1	T-8C	R		R	R	L533P			
1[a]	L533P	S315T1	WT	R	R	WT	S315T1	WT	S	R	R	R	WT L533P			
3[c]	MUT	S315T1	WT	R	R	526-529	S315T1	WT	R	R	R	R	H526L			
1	MUT	S315T1	WT	R	R	530-533	S315T1	WT	R	R	R	R	S531L			
1	MUT	S315T1	WT	R	R	510-517	S315T1	WT	R	R	R	R	Q513K			
1[a]	MUT	WT	WT	R	S	H526D S531L	WT	WT	R	S	R	R	H526D S531L	WT	WT	WT
1[c]	MUT	WT	WT	R	S	513-519	S315T1	-15 to -17	R	R	R	R	D516Y	S315T1	C-17T	

DST, drug susceptibility testing; RMP, rifampicin; INH, isoniazid; WT, wildtype; S, susceptible; R, resistant; IND, indeterminate; MUT, unidentified mutation; when wildtype bands were missing in the Genotype MTBDRplus assay the base-pair region is indicated (i.e. 526-529) ;

[a]mixed cultures; [b]one *M. bovis* ssp. *bovis* and one *M. bovis* var. BCG included, [c]one *M africanum* included,

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