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1 **Antibiograms and Molecular Characterization of Drug Resistance of**
2 ***Mycobacterium abscessus* complex from Patients with Multidrug-Resistant**
3 **Pulmonary Tuberculosis (MDR TB) Infection**

4

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10

11 **Running Head:** Drug Resistance of *Mycobacterium abscessus* complex

12

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16 **Keywords:** Antibiograms, Molecular characterization, *Mycobacterium abscessus*, *Mycobacterium massiliense*

17

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18 **ABSTRACT**

19 In the Philippines, acid fast bacilli positive sputum samples commonly treated as
20 TB due to *Mycobacterium tuberculosis* (MTB) complex. However, *Mycobacterium*
21 *abscessus* (MAB) complex is often found in MTB cultures, or in patients confirmed
22 negative for TB through sputum microscopy and culture. Hence, patients
23 with MAB infections are mistakenly prescribed six-month anti-TB treatments. In this
24 study, MAB complex isolates from MDRTB patients were identified and further
25 sub-speciated using the *mass* 3210 gene. Antimicrobial susceptibility was tested using
26 broth microdilution and resistance genes *erm*(41), *rrs, rrl*, *gyr*A, and *gyr*B were
27 studied for mutations. Majority were susceptible to amikacin, azithromycin,
28 clarithromycin, and moxifloxacin [MAB: 100%, 100%, 100%, 81.8%, respectively; *M.*
29 *massiliense* (MAM): 100%, 100%, 100%, 60%, respectively]. 50% MAM and 63.6%
30 MAB were susceptible to cefoxitin; 60% MAM and 45.5% MAB were susceptible to
31 ciprofloxacin; 72.7% MAB, and 10% MAM were susceptible to doxycycline. Inducible
32 resistance to azithromycin and clarithromycin was found in 27.3% MAB and 30%
33 MAM. 42.9% MAB complex isolates were MDR. Macrolide resistant MAB and
34 MAM had T28 sequevar, showing functional *erm*(41) responsible for inducible
35 resistance. Unexpectedly, full length *erm*(41) was found in MAM. The*rrl* gene in these
36 isolates showed no point mutations, indicating T28 sequevar as cause of inducible
37 resistance. All fluoroquinolone resistant isolates showed Ala-83 in *gyr*A
38 fluoroquinolone resistant-dependent region (QRDR) and Arg-447 and Asn-464
39 in *gyr*B QRDR. These are associated with resistance to the drug.

40

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41 **INTRODUCTION**

42 In the Philippines, one of the most common rapidly growing mycobacteria (RGM) from
43 patients with multiple drug resistant tuberculosis (MDR-TB) infection is *M. abscessus*
44 complex. This non-tuberculous *Mycobacterium* (NTM) species is usually found in TB
45 culture, either growing alongside with *M. tuberculosis* or after the patient has been
46 confirmed negative for pulmonary tuberculosis through direct sputum smear
47 microscopy (DSSM) and TB culture. Pulmonary diseases caused by the *M. abscessus*
48 complex are extremely hard to manage for these species are found to be resistant to
49 anti-tuberculous agents. Patients with *M. abscessus* infection do not receive appropriate
50 treatment for they are considered to have chronic TB and MDR-TB. As a result,
51 patients are prescribed a six-month anti-TB treatment which is not appropriate for *M.*
52 *abscessus* infections. Interestingly, there is no known study in the Philippines regarding
53 the antimicrobial resistance profile of *M. abscessus* complex. NTM studies are
54 overshadowed by pulmonary TB research studies and therefore, clinicians are not
55 guided on the treatment for NTM infections. Moreover, detection of NTM species
56 is not part of the routine procedure in the clinical microbiology laboratory. Treatment
57 failure to both first-line anti-TB drugs rifampicin and isoniazid is linked to infection
58 with multidrug-resistant *M. tuberculosis* (MDR-TB). Gler *et al.* (2012) showed very
59 high rates (83% to 97%) of MDR-TB infections in the Philippines in 2012. Because
60 MDR TB is often associated with the occurrence of *M. abscessus* complex in clinical
61 samples, the present study aimed to characterize local isolates of *M. abscessus* complex
62 from patients with multidrug-resistant pulmonary tuberculosis in terms of their
63 antimicrobial susceptibility profiles and determine the genetics of the antibiotic

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64 resistance. More specifically, this study aimed to determine the phenotypic
65 characteristics of *M. abscessus* complex using colony morphology, growth rate, and
66 pigmentation; to determine subspecies of the *M. abscessus* complex isolates using
67 amplification and sequencing of the *mass_3210* gene; to assess susceptibility of *M.*
68 *abscessus* complex to antimicrobials (clarithromycin CLR, azithromycin AZM,
69 amikacin AMK, ciprofloxacin CIP, moxifloxacin MXF, cefoxitin FOX, and
70 doxycycline DOX) commonly used against it; and to determine the mechanism of
71 antibiotic resistance by conducting sequence analysis of the drug resistance related
72 genes [*erm*(41), *rrl*, *rrs*, *gyr*A, and *gyr*B] in selected isolates.
73

74 **MATERIALS AND METHODS**

75 **Study Site and Bioethical Clearance**

76 All procedures involving handling of infectious materials (culture, DNA extraction,
77 NTM identification, and drug susceptibility tests) were conducted in the P3 laboratory
78 of the National Center for Pulmonary Research, Lung Center of the Philippines. The
79 study sought approval form the Institutional Ethics Review Board of the Lung Center
80 of the Philippines. The requirement for informed consent was waived since the isolates
81 from the stock cultures to be studied were anonymized, but bioethical clearances from
82 the De La Salle University – Manila, and the Lung Center of the Philippines were
83 procured.
84

85 **Study Isolates and Identification of *Mycobacterium abscessus***

86 The sample size computation for an interval estimate of population

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87 was used to determine the required sample size of *M. abscessus* complex isolates for the study. This is as follows: n= t^2 x p (1-p) / m^2, where

88 *n* is the required sample size, t is the confidence level at 95% (standard value of 1.96),

89 p is the estimated prevalence of NTM, and m is the relative precision (Charan &

90 Biswas, 2013). The prevalence rate used for the sample size computation was from the

91 report of Siapno *et al*. (2016) on a retrospective study of 6,886 specimens from NTM

92 infection in a tertiary hospital in the Philippines, which was 2.28%. The relative

93 precision used was one-fifth of the prevalence which was 0.00456. Since the prevalence

94 rate was small, the precision of 5% seemed to be inappropriate. A conservative choice

95 would be one-fourth or one-fifth of prevalence as the amount of precision in the case

96 of small prevalence (Pourhoseingholi *et al*., 2013). The computed sample size of 20

97 was based on NTM, and the study adapted this as 20 *M. abscessus* complex. One hundred seventeen (117) NTM clinical isolates collected from patients with

98 multiple drug-resistant TB infections and were confirmed MPT64 antigen negative

99 were obtained from the Programmatic Management of Drug Resistant Tuberculosis

100 (PMDT) TB culture located at the National Center for Pulmonary Research, Lung

101 Center of the Philippines from which these 20 isolates identified to be *M. abscessus* complex were taken.

102

103 **Phenotypic characterization of *Mycobacterium abscessus* complex**

104 Freshly grown 5 to 7-day old cultures of *M. abscessus* complex were subjected to

105 phenotypic characterization. Colony morphology was determined by observing the

106 colonies grown on Ogawa medium. There are two possible morphotypes of *M.*

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107 *abscessus* complex, namely: the smooth and rough colonies. Growth rate was also
108 determined by checking the presence of visible colonies on Ogawa media on a daily
109 basis. Pigmentation was determined by observing the color of the colonies on the
110 Ogawa medium. *Mycobacterium abscessus* complex is comprised of non-
111 photochromogens. All isolates were tested using the Ziehl Neelsen method to confirm
112 the presence of acid fast bacilli (AFB) and to check the purity of the culture. More so,
113 Kudoh method was conducted in cultures with contaminants. This involved adding 5%
114 NaOH to the culture of *M. abscessus* complex grown in 7H9 broth in a ratio of 1:1 (v/v)
115 The processed isolates were re-incubated at 37°C for 5 to 7 days. AFB smear was
116 conducted again, to check for purity and to confirm presence of acid-fast bacilli.
117
118 **Identification of subspecies of *Mycobacterium abscessus* complex**
119 One hundred seventeen (117) NTM clinical isolates were screened for the identification
120 of *M. abscessus* and *M. massiliense* isolates. All isolates were grown in Middlebrook
121 7H9 broth enriched with albumin dextrose catalase (ADC). These were incubated at
122 37°C for 5 to 7 days or until turbidity was observed and checked for purity. New stock
123 cultures were prepared in Ogawa butt-slants to serve as working stocks, and in
124 10% glycerol at -70°C.
125
126 For identification, DNA extraction was done on freshly grown
127 5 to 7 day-old cultures using Bio-Rad Chelex resin (Al-Mutairi *et al*., 2011) as follows:
128 A loopful of mycobacterial isolate was suspended in a 200 µL solution containing 40mg
129 Chelex100 and 100 mL of water. The resulting material was kept at 95°C for 20

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130 minutes. It was centrifuged for 15 minutes at 12,000 x g. The supernatant was
131 transferred to a sterile Eppendorf® tube and was used as the source of DNA for the
132 one-step multiplex PCR assay. The one-step multiplex PCR assay designed by Chae *et*
133 *al.* (2017) was used (1) to discriminate pan-mycobacterial from non-mycobacterial
134 species by amplifying the16S rRNA gene, (2) to distinguish between MTB complex
135 and NTM species in mycobacteria by amplifying the *rv0577*, (3) to identify *M.*
136 *tuberculosis* by amplifying the RD9, (4) to identify *M. tuberculosis* Beijing family by
137 amplifying the *mtbk_20680*, and (5) to identify the five major NTM species and
138 subspecies by amplifying IS*1311* (*M. avium*), DT1 (*M. intracellulare*), *mass_3210* (*M.*
139 *abscessus* and *M. massiliense*), *mkan_rs12360* (*M. kansasii*).
140
141 The PCR mixture was comprised of 1) 2x Prime TAq Premix (Genet Bio., Ltd.
142 Daejeon, South Korea) containing Prime *Taq* DNA polymerase 1 unit/10ul, 2X reaction
143 buffer, 4mM $MgCl_2$, enzyme stabilizer, sediment, loading dye (pH 9.0), and 0.5 mM
144 of each dATP, dCTP, dGTP, and dTTP; 2) 10 pmole of each of the following forward
145 and reverse primers of *rv0577*F (5'-GAG ATA CTC GAG TGG CGA A-3') , *rv0577*R
146 (5'-CAA CGC GAC AAA CCA CCT AC-3'), *DT*1F (5'-AAG GTG AGC CCA GCT
147 TTG AAC TCC A-3'), *DT*1R (5'-GCG CTT CAT TCG CGA TCA TCA GGT G-3'),
148 mtbk_*20680*F (5'-TTA TGC CAG AAA TAC ACC CGC G-3'), mtbk_*20680*R (5'-
149 AAT CGC GGG CTT GTG GCT AC-3'), 16S rRNAF (5'-GAG ATA CTC GAG TGG
150 CGA AC-3'), 16S rRNAR (5'-CAA CGC GAC AAA CCA CCT AC-3'), RD9F (5'-
151 GTG TAG GTC AGC CCC ATC C-3'), RD9R (5'-GTA AGC GCG TGG TGT GGA-
152 3'), IS*1311*F (5'-TCG ATC AGT GCT TGT TCG CG-3'), IS*1311*R (5'-CGA TGG

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153 TGT CGA GTT GCT CT-3'), *mass_3210*F (5'-GCT TGT TCC CGG TGC CAC AC-
154 3'), *mass_3210*R (5'-GGA GCG CGA TGC GTC AGG AC-3'), *mkan_rs12360*F (5'-
155 ACA AAC GGT GTG TCG CAA TGT GCC A-3'), and *mkan_rs12360*R (5'-TGT
156 CGA GCA GAC GTT CCA GGA CGG T-3'), respectively; 3) 2 μl DNA template;
157 and 4) sterile deionized distilled water. The cycling condition included an initial
158 denaturation step at 95°C for 10 minutes, then 30 cycles at 96°C for 45 seconds, 61.5°C
159 for 45 seconds, and 72°C for 40 seconds, followed by 72°C for 10 minutes for the final
160 extension. The amplicons were analyzed using 2% gel for 40 minutes at 100 V in 0.5X
161 TBE buffer. The gels were visualized using the Bio-Rad ImageLab gel reader (Bio-Rad
162 Laboratories Inc. USA).
163
164 **Antimicrobial Susceptibility Testing**
165 The mycobacterial isolates were cultured on Ogawa medium at 37°C for 5 to 7 days.
166 Suspensions were prepared by aseptically sweeping the confluent portion of growth on
167 the Ogawa medium with a sterile loop. Growth on the sterile loop was transferred to
168 4.5 mL of sterile water containing glass beads (7 to 10 3-mm beads) until the turbidity
169 matched the 0.5 McFarland standard with an approximate organism density of 1×10^5
170 colony forming units ml^{-1} (CFU/mL). The final inoculum with 5×10^2 CFU/mL was
171 prepared by transferring 50 μl of the suspension to a tube containing 10 ml of cation
172 adjusted Mueller-Hinton broth (CLSI M24-A2, 2011).
173
174 Antimicrobial susceptibility testing of *M. abscessus* complex isolates was performed
175 using the gold standard assay recommended by the Clinical Laboratory Standards

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176 Institutes (CLSI M24-A2, 2011) which is the microdilution technique. BBL cation
177 adjusted Mueller-Hinton (CAMH) Broth (BD, Franklin Lakes, NJ, USA) with 5%
178 OADC was used as the medium for the tests. Tests on the mycobacterial strains were
179 conducted in 96-well microplates. The broth microdilution method is traditionally set
180 up as two-fold dilutions. The following working ranges were used for the mycobacteria:
181 the concentration range for amikacin, cefoxitin, and azithromycin was 0.25 to 256
182 μg/mL; the concentration range for clarithromycin, ciprofloxacin, doxycycline, and
183 moxifloxacin was 0.0625 to 64 μg/mL (CLSI M24-A2, 2011). All antimicrobial drugs
184 used for the antimicrobial susceptibility test were procured from Sigma Aldrich
185 (Merck Group, USA). To determine the amount of antimicrobial agent
186 powder needed for a standard solution, the formula outlined in the CLSI M24-A2, 2011
187 was used.
188
189 The thoroughly mixed antimicrobial dilutions (100 μl) were dispensed into each well,
190 except for the 11th well, which is a drug-free well for bacterial growth control. The 1st
191 well contained 200 μl of CAMHB and served as the negative control. A total of 100
192 μl of the final inoculum was dispensed in the second well up to the 11th well. The final
193 volume in each well was 200 μl. Reference strain of antimicrobial susceptible *M.*
194 *peregrinum* ATCC® 700686 was tested along with the test clinical isolates to serve as
195 the quality control. The inoculated 96-well microplate were sealed and incubated at
196 37°C. The MIC values for amikacin, cefoxitin, doxycycline, ciprofloxacin,
197 moxifloxacin, azithromycin and clarithromycin were read on the third day after the
198 inoculum was added. The MICs of azithromycin and clarithromycin for isolates

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199 showing susceptible results on the third day were read again on the 7th day and 14th day
200 to determine the presence of inducible resistance to the macrolides (Nie *et al.,* 2014).
201 Inducible resistance to macrolides was concluded once the mycobacterial strains
202 showed azithromycin or clarithromycin susceptibility on the third day and resistance
203 on the 7th or 14th day (Lee *et al*., 2014). Thirty microliters of a freshly prepared 0.01%
204 of 10X resazurin (Accumed International, Westlake, Ohio) reagent were added to all
205 wells in the microplate. A blue color in the well was interpreted as no growth, while a
206 pink color was scored as growth. The MIC was defined as the lowest drug concentration
207 which prevented the color change from blue to pink (Franzblau *et al.,* 1998). Each test
208 was done in triplicate.
209

210 **PCR amplification of *rrl, erm*(41)*, rrs, gyrA,* and *gyrB* genes**

211 Sequencing of *rrl* and *erm*(41) genes was conducted to compare and analyze the
212 characteristics of the susceptible and resistant *M. abscessus* strains to AZM and CLR,
213 respectively. The primer sets are as follows: *erm*(41)F (5'-GAG CGC CGT CAC AAG
214 ATG CAC A-3'), *erm*(41)R (5'-GAC TTC CCC GCA CCG ATT CCA C-3'), *rrl*F (5'-
215 GTA GCG AAA TTC CTT TGT CGG-3'), and *rrl*R (5'-TTC CCG CTT AGA TGC
216 TTT CAG-3') (Nash *et al*., 1995; Nash *et al*., 2009). The primer sets for the
217 amplification of the amikacin resistance-related gene are *rrs*F (5'-CAG TAC AGA
218 GGG CTG CGA ACG-3') and *rrs*R (5'-AAG GAG GTG ATC CAG CCG CA-3')
219 (Prammananan *et al.,* 1998). To characterize resistant *M. abscessus* strains to CIP and
220 MXF, sequence analysis of the fluoroquinolone resistant-dependent region (QRDR) of
221 the *gyrA* and *gyrB* genes was conducted. The primer sets are: *gyr*AF (5'-GGG CAT

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222 CTA AAG CCG CTG AGA-3'), *gyr*AR (5'-GAC GAT GGC GCG CTG ACG T-3'),
223 *gyr*BF (5'-GCA GAT GCT AAA ACG GTT GTG A-3') and *gyr*BR (5'-CTC GTA
224 AGT ACG ACG GCA CAA-3') (Guillemin *et al.,* 1998).
225
226 **RESULTS and DISCUSSION**
227
228 **Phenotypic Characterization of *Mycobacterium abscessus* complex**
229 The *M. abscessus* complex isolates were characterized based on colony
230 morphology, growth rate, and pigmentation. The isolates tended to aggregate and form
231 biofilms on the third day of incubation in 7H9 broth medium incubated at 37$_o$C.
232 Biofilms were visible both on the surface and at the bottom of the tubes. The enriched
233 cultures of *M. abscessus* complex from 7H9 medium were grown in Ogawa butt slants
234 to determine the proportion of smooth and rough colonies. Growth on the surface of
235 the Ogawa medium was observed on the third day of incubation at 37$_o$C.
236
237 The phenotypic characteristics of the 21 *M. abscessus* complex isolates are shown in
238 Figures 1 and 2, and Table 1. Smooth colonies of *M. abscessus* and *M. massiliense*
239 were moist, shiny, and round, while the rough colonies were dry, waxier, and wrinkled.
240 The smooth colony of *M. abscessus* complex expresses glycopeptidolipid (GPL) on its
241 cell wall and forms biofilms, while rough colony of *M. abscessus* complex does not
242 form biofilms due to minimal amounts of GPL (Howard *et al*., 2006; Byrd & Lyons,
243 1999). Moreover, rough colony of *M. abscessus* is associated with virulence compared
244 to the smooth colony of *M. abscessus*. In the present study, a total of 7 isolates (63.6%)

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245 of *M. abscessus* and 3 (30.0%) *M. massiliense* isolates were characterized to have
246 smooth colonies, while 4 (36.4%) *M. abscessus* and 7 (70.0%) *M. massiliense* isolates
247 produced rough colonies. All *M. abscessus* and *M. massiliense* isolates were buff –
248 colored and were categorized as nonphotochromogens, i.e., they were non-pigmented
249 whether they were grown in the dark or in the presence of light. All (100%, 21/21)
250 isolates that grew in Ogawa medium were acid fast. NTM appears as shorter rods
251 compared to *M. tuberculosis* when viewed microscopically.
252
253 **Subspecies identification of *Mycobacterium abscessus* complex isolates**
254 The subspecies of the 117 clinical isolates that were found to be negative for MPT64
255 were identified using multiplex PCR. Among the 117 clinical isolates, 11 (9.4%) were
256 identified as *M. abscessus* and 10 (8.5%) were *M. massiliense* (Figure 4). The
257 remaining isolates were identified as *M. tuberculosis* (15%, 17/114), other NTM
258 species (53.8%, 63/117), and other microorganisms (14%, 16/114). It is also
259 noteworthy that all 117 isolates were found negative using the MPT64 antigen kit, but
260 some were identified as *M. tuberculosis* using the one-step multiplex PCR assay. These
261 results may infer that mixed colonies of *M. tuberculosis* and NTM might be present in
262 the stock cultures from the bank specimens. Purification was conducted on all identified
263 *M. abscessus* complex isolates. Positive controls of *M. abscessus* and *M. massiliense,*
264 which were clinical isolates from the bank specimens in the National Center for
265 Pulmonary Research – TB laboratory that were previously identified by reverse blot
266 hybridization assay were tested along with the *M. abscesus* complex test isolates.
267

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268 All mycobacteria showed the 506bp band corresponding to the 16S rRNA internal
269 control which was specific only to all mycobacterial species. *M. abscessus* isolates
270 showed the expected 310bp band and *M. massiliense* showed the 1145bp band using
271 the primers for *mass*_3210. The *mass*_3210 is a specific gene previously identified
272 and used to discriminate between *M. abscessus* complex species (Chae *et al.,* 2017).
273 Further analysis of the *mass*_3210 aligned sequences of representative isolates showed
274 two clustered groups: MAB01-MAB05 and MAS01-MAS05 (Figure 4). This result
275 implies that MAB01-MAB05 isolates clustered together, showing that they belonged
276 to one subspecies (*M. abscessus*). On the other hand, MAS01-MAS05 isolates grouped
277 together, showing that they belonged to *M. massiliense* subspecies.
278

279 **Drug-resistance Profile of *Mycobacterium abscessus* complex**

280 Results of the antimicrobial susceptibility assay of the *M. abscessus* complex are shown
281 in Table 2. The present study showed that amikacin was the most active agent against
282 all *M. abscessus* and *M. massiliense* isolates with an MIC of <1μg/ml for both groups.
283 These are in conformity with the results of the study of Nie *et al.* in 2014 which showed
284 that the susceptibility rate of *M. abscesuss* to AMK was 98%. Likewise, Park *et al.*
285 (2008) reported a 99% susceptibility rate of *M. abscessus* to AMK, while Kim *et al.*
286 (2015) reported the susceptibility rate of *M. abscessus* and *M. massiliense* to AMK as
287 91.2% and 100%, respectively. The susceptibility of all isolates to amikacin in the
288 present study may be attributed to the current treatment regimen for MDR-TB in the
289 country which is comprised of kanamycin and not amikacin. Amikacin resistant
290 isolates are not positively selected for to survive and thus are not disseminated.

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291

292 On the other hand, FOX was shown to be less potent *in vitro* against *M. abscessus* and
293 *M. massiliense* isolates compared to amikacin. Although there were no *M. abscessus*
294 isolates that were resistant to the drug, and a low 10% of the *M. massiliense* were
295 resistant to the agent, 63.6% of the *M. abscessus* and 50% of the *M. massiliense* isolates
296 were only moderately susceptible to cefoxitin. The remaining 36.4% and 40% of the
297 *M. abscessus* and *M. massiliense* isolates were susceptible to it. The results are
298 comparable to those of Nie *et al.* (2014) that showed 0% resistant *M. abscessus* isolates,
299 while 53% were moderately susceptible. Koh *et al.* (2010) likewise showed no resistant
300 isolates of *M. abscessus* and only 1% resistance rate in *M. massiliense* against FOX.
301 Lee *et al.* (2015) reported resistance rate of 1.3% in *M. abscessus* subsp. *massiliense*
302 and 0% for *M. abscessus* subsp. *abscessus* against FOX.

303

304 Susceptibility rates of *M. abscessus* complex isolates in the present study showed that
305 MXF has better *in vitro* activity against *M. abscessus* (81.8% susceptible) and *M.*
306 *massiliense* (60% susceptible) compared to CIP against *M. abscessus* (9.1%
307 susceptible) and *M. massiliense* (20% susceptible). This is consistent with other studies
308 showing that MXF has better *in vitro* activity against *M. abscessus* and *M. massiliense*
309 compared to CIP (Kim *et al.,* 2015; Lee *et al.,* 2014; Koh *et al.,* 2010). On the other
310 hand, DOX was found to be more potent against *M. abscessus* (72.7% susceptible)
311 isolates compared to its activity against *M. massiliense* (10% susceptible). The
312 resistance rate of *M. massiliense* (60%) in the present study is compatible with the
313 findings of Koh *et al.* (73%) and Lee *et al.* (73.4%).

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314

315 Macrolides, specifically, AZM and CLR are the drugs of choice for the treatment of
316 infections caused by the *M. abscessus* complex. The present study reported 100%
317 susceptibility rate for both *M. abscessus* and *M. massiliense* isolates on the 3rd day of
318 incubation. However, inducible resistance to both CLR and AZM were found in *M.*
319 *abscessus* MAB04 with MICs of >16ug/ml for both drugs on the 7th day of incubation,
320 while *M. abscessus* MAB07 showed inducible resistance to AZM with an MIC of
321 16µg/ml on the 7th day, and inducible resistance to CLR on the 14 th day (MIC
322 16µg/ml). *Mycobacterium abscessus* isolate MAB09 showed inducible resistance to
323 both CLR (>16µg/ml) and AZM (>16µg/ml) on the 14th day of incubation. Inducible
324 resistance in *M. abscessus* was expected since they have a functional erm(41) gene
325 (Kim *et al.,* 2015; Koh *et al.,* 2010; Nash *et al.,* 2009). On the other hand, three *M.*
326 *massiliense* isolates (MAS04: >16µg/ml, MAS06: >16µg/ml, MAS10: >16µg/ml)
327 exhibited inducible resistance to CLR and AZM on the 14th day. This is contrast with
328 previous studies (Kim *et al.,* 2015; Koh *et al.,* 2010) which demonstrated that inducible
329 resistance to CLR was only present in *M. abscessus* and not in *M. massiliense.*
330 Nevertheless, our results support the findings of Li *et al*. (2017) and Shallom *et al.*
331 (2013) showing that *M. massiliense* exhibited an inducible resistance to CLR. Lastly,
332 the MIC of the test drugs for the susceptible reference *M. peregrinum* ATCC 700686
333 used for quality control were all in accordance with the tentative quality control ranges
334 for rapidly growing mycobacteria as indicated in the CLSI M24-A2 (2011) guidelines.
335
336 The resistance phenotypes of *M. abscessus* complex isolates are summarized in Table

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337 3. Overall, 42.9% (9/21) of the *M. abscessus* complex isolates were found MDR; 14.3%
338 of which were *M. abscessus* isolates and 28.6% of were *M. massiliense* isolates. MDR
339 is defined as resistance to at least one agent in three or more antimicrobial categories
340 (Magiorakos *et al.,* 2012).
341
342 Although the MDR percentage obtained in the study is lower than the 97.2% MDR
343 report of Candido *et al.* (2104), where *M. abscessus* complex isolates were found to be
344 resistant to five or more antimicrobial agents, the emergence of multidrug-resistant *M.*
345 *abscessus* complex isolates in the country is of serious concern which impacts on the
346 current treatment regimen given to patients with *M. abscessus* infection. This calls for
347 the need to conduct antimicrobial susceptibility testing of clinical isolates of *M.*
348 *abscessus* complex for better management of the patients, and for the prevention of the
349 selection for resistant strains to survive and be disseminated.
350
351 **Molecular Characterization of Drug-resistance of *Mycobacterium abscessus***
352 **comple**x
353 In the present study, the *erm*(41) gene of all the *M. abscessus* and *M. massiliense*
354 isolates with inducible resistance was amplified. All three *M. abscessus* isolates
355 (MAB04, MAB07, MAB09) showing inducible resistance to CLR and AZM were
356 found to have a full-length *erm*(41) gene of 892bp. These isolates with the full-length
357 *erm*(41) gene have a T28 sequevar which is associated with inducible resistance to
358 macrolides. Such resistance can affect the current treatment regimen of a patient, thus
359 contributing to the hurdles towards successful treatment. Interestingly, all three *M.*

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360 *massiliense* isolates (MAS04, MAS06, MAS10) with an MIC of >16ug/ml harbored a
361 full-length *erm*(41) gene that is present only in *M. abscessus* (Nash *et al.,* 2009),
362 instead of the expected truncated *erm*(41) gene . This finding supports the results of
363 other studies (Lipworth *et al.,* 2018; Shallom *et.,* 2013), which also reported the
364 presence of a functional *erm*(41) gene in *M. massiliense* leading to a resistant
365 phenotype. The mutation patterns of the macrolide-resistant *M. abscessus* complex
366 isolates are summarized in Table 4. Caution should thus be practiced in using the
367 *erm*(41) gene in identifying subspecies of *M. abscessus* complex.
368
369 Among the six macrolide-resistant isolates, MAB4 showed the most number of
370 nucleotide substitutions in the *erm*(41) gene. MAB4 is also the only isolate that showed
371 inducible resistance to both azithromycin and clarithromycin earliest on the 7th day.
372 Such polymorphisms include nucleotide substitutions in positions A238G, C253A,
373 A255T, and A312C, all of which lead to missense mutations. Additionally, all isolates
374 harbored the T28 sequevar which is associated with inducible resistance to macrolides.
375 Aside from harboring a thymine at position 28 (T28), the remaining isolates, except for
376 MAB4 and MAB7, showed base substitution at position 168 (G168C). Nevertheless,
377 this only resulted in silent mutation. In addition, base substitution at position 255
378 (A255G) was found in all isolates, except for MAB4, which also resulted to a silent
379 mutation, thus no observable effect on the organism's phenotype.
380
381 The 836 bp amplicons of the *rrl* gene of macrolide resistant isolates of *M. massiliense*
382 did not show mutations in positions 2057, 2058 and 2059 which are commonly

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383 associated with acquired resistance (Wallace *et al*., 1996; Bastian *et al*., 2011; Maurer
384 *et al.,* 2014). Hence, the macrolide resistance shown in these isolates in the study can
385 be attributed to its T28 sequevar in the *erm*(41) gene. There were three *M. abscessus*
386 isolates that showed inducible resistance to macrolides which is attributed to the T28
387 sequevar of the *erm*(41) gene.
388 Sequence analysis of the *gyr*A quinolone resistance-determining region (QRDR)
389 revealed only one nucleotide substitution(T252C) present in *M. abscessus* complex
390 isolates, while four nucleotide substitutions (T1443C, C1458T, C1479A, C1494T)
391 were observed in the *gyr*B QRDR (Table 5). Nevertheless, all observed nucleotide
392 substitutions in the *gyr*A and *gyr*B QRDR only caused silent mutations, which do not
393 cause observable effect on the organism's phenotype. However, it was also observed
394 that all fluoroquinolone resistant isolates of *M. abscessus* complex showed the presence
395 of an alanine residue at position 83 (Ala-83) in the *gyr*A QRDR. The presence of Ala-
396 83 is different from those reported in susceptible bacteria harboring a serine residue at
397 position 83 (Ser-83) (Yoshida *et al.,* 1990). Moreover, all ciprofloxacin resistant
398 isolates harbored an arginine residue at position 447 (Arg-447), and an asparagine
399 residue at position 464 (Asn-464) in the *gyr*B QRDR. These amino acid substitutions
400 are different from those found in susceptible bacteria harboring a lysine at position 447
401 (Lys-447) and a serine at position 464 (Ser-464_ (Yoshida *et al.,* 1991). These results
402 are in accordance with other studies (Esfahani *et al.,* 2016; de Moura *et al.,* 2012;
403 Guillemin *et al.,* 1998; Guillemin *et al.,* 1995;), showing that substitutions of amino
404 acids at these positions are associated with acquired resistance to fluoroquinolones. It
405 is hypothesized that the presence of these amino acids could decrease the interaction

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406 between the quinolone drug and the gyrase A and B subunit-DNA complex (Guillemin
407 *et al.,* 1998).
408
409 To our knowledge, this is the first study to assess the drug resistance profile including
410 determination of the genetic mechanisms of drug resistance of *M. abscessus* complex
411 in the Philippines. Therefore, the findings of this study presented a potential area of
412 interest about nontuberculous mycobacteria (NTM) that need to be explored.
413
414 Based on the findings of the study, correct identification of *M. abscessus* complex
415 subspecies is important because their responses to anti-TB medications vary from one
416 subspecies to another. Isolation and identification of NTM species, which are not parts
417 of the routine procedure in most clinical microbiology laboratory in the Philippines is
418 important to understand the issues on NTM in clinical samples.
419
420
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425

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558 **Figure Legends**

559 **Figure 1.** Biofilm formation of *M. abscessus* complex in 7H9 medium (A). Colony
560 morphology of *M. abscessus* complex isolates showing two morphotypes: rough and
561 smooth colonies (B). All isolates are nonphotochromogens (non-pigmented).

562

563 **Figure 2.** Positive AFB smear result under 1000x magnification, with immersion oil.

564

565 **Figure 3.** One-step Multiplex PCR Assay results using primers for *mass*_3210 gene.
566 Molecular marker (100 plus bp); Ctrl MAB: *M. abscessus* control (310bp *mass*_3210
567 amplicon) and the *Mycobacterium*-specific16S rRNA internal control (506bp); Ctrl
568 MAS: *M. massiliense* control (1145bp *mass*_3210 amplicon) and the *Mycobacterium*
569 specific 16S rRNA internal control (506bp). MAS = *M. massilience;* MAB = *M.*
570 *abscessus.*

571

572 **Figure 4.** Neighbor-joining tree based on *mass*_3210 sequences. Dendrogram showing
573 the clustering of *M. abscessus* isolates (MAB01-MAB05) and *M. massiliense* isolates
574 (MAS01-MAS05) together with *mass*_3210. The *IS*_1311 sequence which is specific
575 to *M. avium* complex (MAC) was used as the outgroup.

576

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577 **Table 1.** Phenotypic characteristics of *M. abscessus* and *M. massiliense* isolates from patients with MDR TB infections.

Study ID	AFB Smear	Growth Rate	Pigmentation	Colony Morphology
MAB01	+	Day 3	buff	smooth
MAB02	+	Day 3	buff	rough
MAB03	+	Day 3	buff	rough
MAB04	+	Day 3	buff	smooth
MAB05	+	Day 3	buff	smooth
MAB06	+	Day 3	buff	smooth
MAB07	+	Day 3	buff	rough
MAB08	+	Day 3	buff	rough
MAB09	+	Day 3	buff	smooth
MAB10	+	Day 3	buff	smooth
MAB11	+	Day 3	buff	smooth
MAS01	+	Day 3	buff	rough
MAS02	+	Day 3	buff	rough
MAS03	+	Day 3	buff	rough
MAS04	+	Day 3	buff	rough
MAS05	+	Day 3	buff	smooth
MAS06	+	Day 3	buff	smooth
MAS07	+	Day 3	buff	rough
MAS08	+	Day 3	buff	smooth
MAS09	+	Day 3	buff	rough
MAS10	+	Day 3	buff	rough

584 MAB = *M. abscessus*; MAS = *M. massiliense*

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585 **Table 2.** Antimicrobial susceptibility test results of *M. abscessus* and *M. massiliense*
586 using broth microdilution method against seven (7) antimicrobial drugs.

Antimicrobial Drugs	*M. abscessus* subspecies (n)	Results read on	MIC range (ug/ml)	Susceptible No. (%)	Moderately Susceptible No. (%)	Resistant No. (%)
Amikacin	*M. abscessus* (n=11)	Day 3	<1	11 (100%)	0	0
	M. massiliense (n=10)		<1	10 (100%)	0	0
Cefoxitin	*M. abscessus* (n=11)	Day 3	4-64	4 (36.4%)	7 (63.6%)	0
	M. massiliense (n=10)		16-128	4 (40.0%)	5 (50.0%)	1 (10.0%)
Ciprofloxacin	*M. abscessus* (n=11)	Day 3	0.125-8	1 (9.1%)	4 (36.4%)	6 (54.5%)
	M. massiliense (n=10)		0.25-16	2 (20.0%)	6 (60.0%)	2 (20.0%)
Moxifloxacin	*M. abscessus* (n=11)	Day 3	0.125-2	9 (81.8%)	2 (18.2%)	0
	M. massiliense (n=10)		0.5-16	6 (60.0%)	3 (30.0%)	1 (10.0%)
Doxycycline	*M. abscessus* (n=11)	Day 3	0.25-32	8 (72.7%)	1 (9.1%)	2 (18.2%)
	M. massiliense (n=10)		0.125-16	1 (10.0%)	3 (30.0%)	6 (60.0%)
Azithromycin	*M. abscessus* (n=11)	Day 3	<0.06-1	11 (100%)	0	0
		Day 7	0.125->16	9 (81.8%)	0	2 (18.2%)
		Day 14	0.25->16	7 (63.6%)	1 (9.1%)	3 (27.3%)
	M. massiliense (n=10)	Day 3	<0.06-1	10 (100%)	0	0
		Day 7	0.125-1	10 (100%)	0	0
		Day 14	0.125->16	7 (70%)	0	3 (30%)
Clarithromycin	*M. abscessus* (n=11)	Day 3	<0.06-0.5	11 (100%)	0	0
		Day 7	<0.06->16	9 (81.8%)	1 (9.1%)	1 (9.1%)
		Day 14	<0.06->16	7 (63.6%)	1 (9.1%)	3 (27.3%)
	M. massiliense (n=10)	Day 3	<0.06-2	10 (100%)	0	0
		Day 7	<0.06-2	10 (100%)	0	0
		Day 14	0.25->16	7 (70%)	0	3 (30%)

587 [a] extended incubation up to 14 days to determine inducible resistance to macrolides

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588 **Table 3.** Resistance phenotypes of *M. abscessus* and *M. massiliense* in the study

Study ID	Subspecies Identification	Antimicrobial Agents							MDR*
		AZM	CLR	AMK	FOX	CIP	MXF	DOX	
MAB01	*M. abscessus*	S	S	S	M	R	S	M	+
MAB02	*M. abscessus*	S	S	S	M	M	S	S	-
MAB03	*M. abscessus*	S	S	S	M	S	S	S	-
MAB04	*M. abscessus*	IR	IR	S	M	S	S	S	-
MAB05	*M. abscessus*	S	S	S	M	R	S	S	-
MAB06	*M. abscessus*	S	S	S	M	R	S	S	-
MAB07	*M. abscessus*	IR	IR	S	S	M	S	S	-
MAB08	*M. abscessus*	S	S	S	S	R	S	S	-
MAB09	*M. abscessus*	IR	IR	S	M	R	M	R	+
MAB10	*M. abscessus*	S	S	S	M	R	S	S	-
MAB11	*M. abscessus*	S	S	S	M	M	M	R	+
MAS01	*M. massiliense*	S	S	S	S	M	S	M	-
MAS02	*M. massiliense*	S	S	S	M	S	S	M	-
MAS03	*M. massiliense*	S	S	S	S	S	M	R	-
MAS04	*M. massiliense*	IR	IR	S	M	S	S	M	+
MAS05	*M. massiliense*	S	S	S	M	S	M	R	+
MAS06	*M. massiliense*	IR	IR	S	R	R	M	R	+
MAS07	*M. massiliense*	S	S	S	M	M	M	S	-
MAS08	*M. massiliense*	S	S	S	M	R	R	R	+
MAS09	*M. massiliense*	S	S	S	M	M	S	R	+
MAS10	*M. massiliense*	IR	IR	S	M	M	S	R	+

589 Antimicrobial agents tested AZM (Azithromycin), CLR (Clarithromycin), AMK (Amikacin), FOX
590 (Cefoxitin), CIP (Ciprofloxacin), MXF (Moxifloxacin), DOX (Doxycycline). The letter S is for susceptible,
591 M indicates moderate susceptible, R is for resistant, and IR stands for inducible resistant isolate. The
592 hyphen indicates that the isolate is not MDR while the (+) sign indicates that the isolate is MDR. Multidrug-
593 resistant (MDR) is defined as resistance to at least one agent in three or more antimicrobial categories
594 (Magiorakos *et al.*, 2012).

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Table 4. Single nucleotide polymorphisms (SNPs) in the *erm*(41) gene associated with macrolide-resistant isolates of *M. abscessus* and *M. massiliense*

Study ID	T28 TGG (Trp)	T159 GGT (Gly)	G168 GTG (Val)	T231 GAT (Asp)	A238 ATA (Ile)	G249 GCG (Ala)	C253 CTA (Leu)	A255 CTA (Leu)	A312 CAA (Glu)	A330 ATA (Ile)	T336 AGT (Ser)	A414 CGA (Arg)
MAB4	-	C	-	C	G (Val)	A	A (Ile)	T (Ile)	C (His)	C	C	G
MAS4	-	-	C	-	-	-	-	G	-	-	-	-
MAB7	-	-	-	-	-	-	-	G	-	-	-	-
MAB9	-	-	C	-	-	-	-	G	-	-	-	-
MAS6	-	-	C	-	-	-	-	G	-	-	-	-
MAS10	-	-	C	-	-	-	-	G	-	-	-	-

MAB = *M. abscessus*; MAS = *M. massiliense*. The base substitution site is underlined for each codon sequence. The hyphen indicates that the base is the same in the type strain sequence *Mycobacterium abscessus* ATCC 19977. The type strain *M. abscessus* is intrinsically susceptible but has inducible resistance to macrolides. T28 is associated with inducible-macrolide resistance. Bases of DNA: Adenine (A), Guanine (G), Cytosine (C), and Thymine (T).

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602 **Table 5**. Single nucleotide polymorphisms (SNPs) observed in the quinolone resistance-determining region *of gyr*A and *gyr*B of

603 fluoroquinolone-resistant isolates of *M. abscessus* and *M. massiliense*

Study ID	***gyr*A gene**	***gyr*B gene**			
	T252 TAT (Tyr)	**T1443 GGT (Gly)**	**T1458 TTC (Phe)**	**C1479 CGC (Arg)**	**C1494 AAC (Asn)**
MAB1	C	C	T	-	-
MAB5	C	C	T	A	-
MAB6	C	C	T	-	-
MAB8	C	C	T	-	-
MAB9	-	-	-	-	T
MAS6	-	-	-	-	T
MAB10	C	C	T	-	-
MAS8	C	C	T	-	-

604 MAB = *M. abscessus*; MAS = *M. massiliense*. The base substitution site is underlined for each codon

605 sequence. The hyphen indicates that the base is the same in the type strain sequence *Mycobacterium*

606 *abscessus* ATCC 19977. Bases of DNA: Adenine (A), Guanine (G), Cytosine (C), and Thymine (T).

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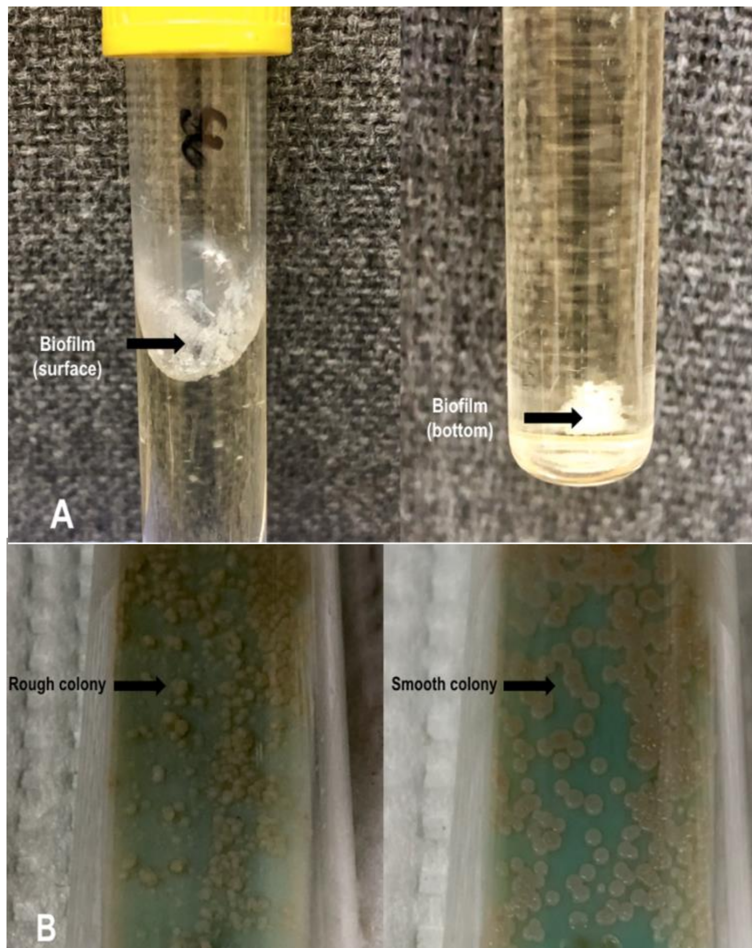

607 **Figure 1.** Biofilm formation of *M. abscessus* complex in 7H9 medium (A). Colony
608 morphology of *M. abscessus* complex isolates showing two morphotypes: rough and
609 smooth colonies (B). All isolates are nonphotochromogens (non-pigmented).

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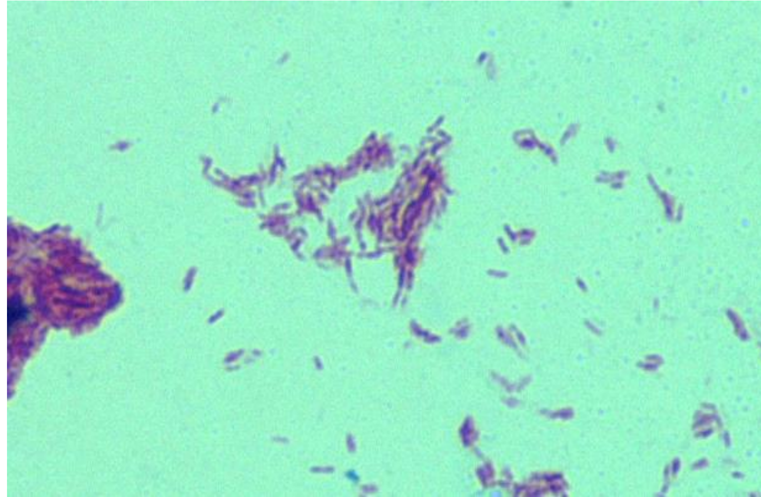

610 **Figure 2.** Positive AFB smear result under 1000x magnification.

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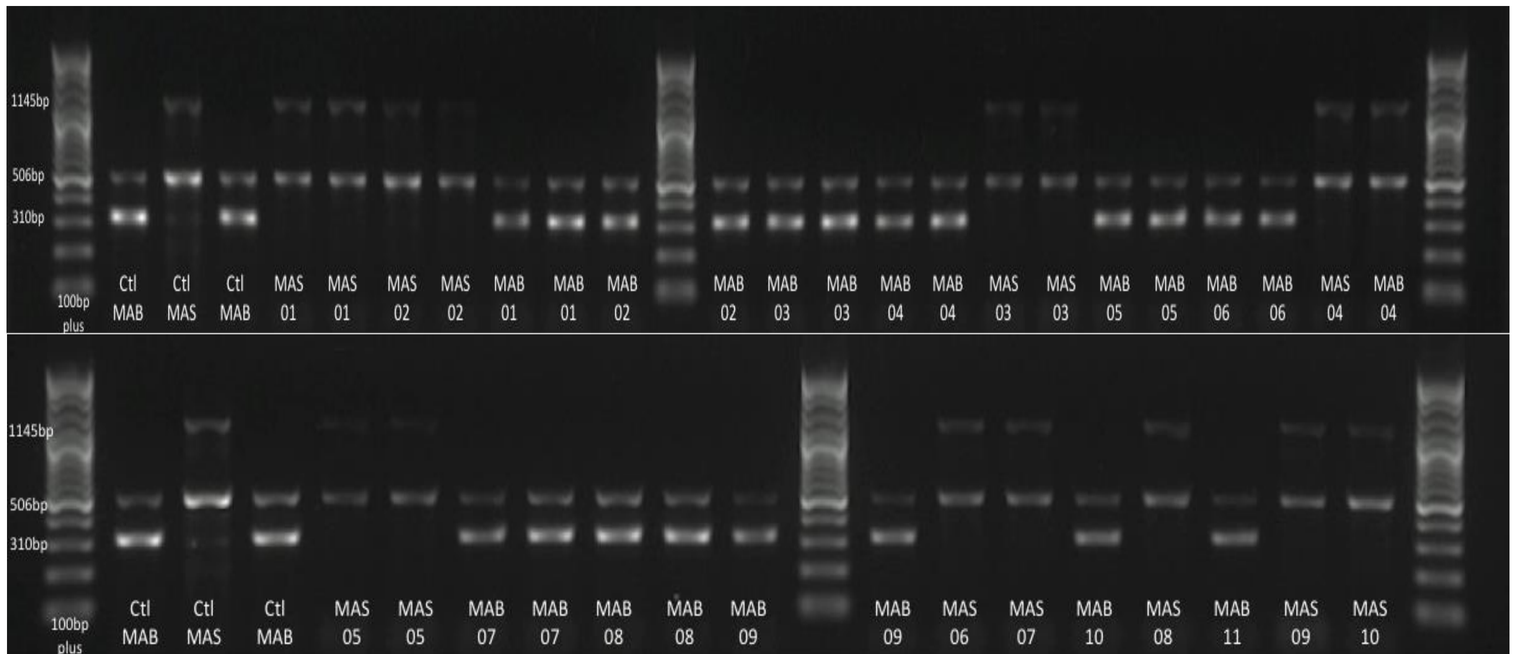

611 **Figure 3.** One-step Multiplex PCR Assay results using primers for *mass*_3210 gene.
612 Molecular marker (100 plus bp); Ctrl MAB: *M. abscessus* control (310bp *mass*_3210
613 amplicon) and the *Mycobacterium*-specific16S rRNA internal control (506bp); Ctrl
614 MAS: *M. massiliense* control (1145bp *mass*_3210 amplicon) and the *Mycobacterium*-
615 specific 16S rRNA internal control (506bp). MAS = *M. massilience;* MAB = *M.*
616 *abscessus.*

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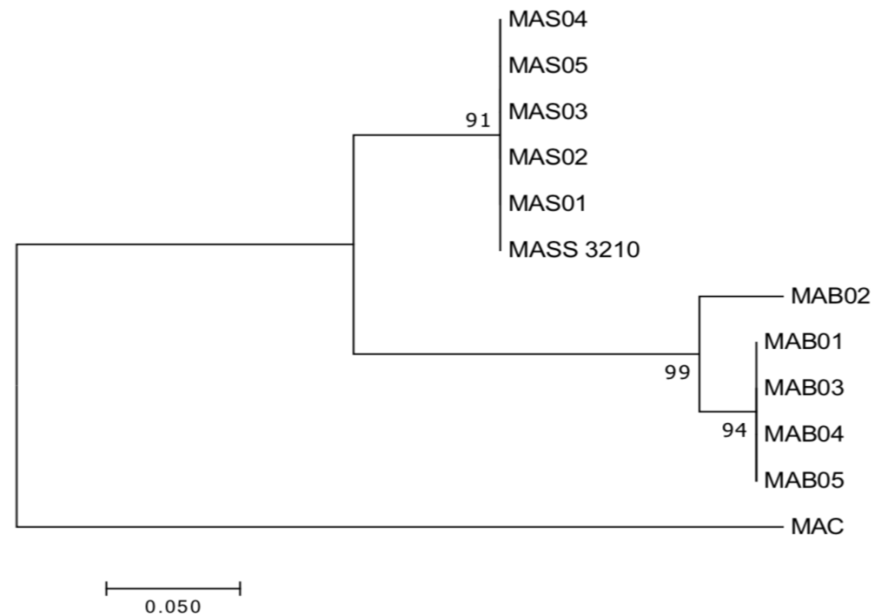

617 **Figure 4.** Neighbor-joining tree based on *mass*_3210 sequences. Dendrogram showing
618 the clustering of *M. abscessus* isolates (MAB01-MAB05) and *M. massiliense* isolates
619 (MAS01-MAS05) together with *mass*_3210. The *IS*_1311 sequence which is specific
620 to *M. avium* complex (MAC) was used as the outgroup.

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