1   <u>**Short Communication**</u>

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3   **Seasonal variation in the performance of QuantiFERON-TB Gold In-Tube**

4   **assays used for the diagnosis of tuberculosis infection**

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Running title: Seasonal variation in performance of QFT-GIT assays

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Key words: tuberculosis, interferon-gamma release assay, performance,

indeterminate, seasonality

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Word count: 1645 words

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46   **ABSTRACT**

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48   This study aimed to determine whether there are seasonal changes in the

49   performance of QuantiFERON-TB Gold In-Tube (QFT-GIT) assays, an interferon-

50   gamma release assay widely used for the diagnosis of tuberculosis infection.

51   Results of 31,932 QFT-GIT assays performed at a large independent, accredited

52   diagnostic service provider in London, UK over a 4.5-year-period were analysed. The

53   proportion of positive results was significantly lower in autumn (14.8%) than in spring

54   (16.0%; p=0.0366) and summer (17.5%; p<0.0001), but similar to winter (15.2%;

55   p=0.4711). The proportion of indeterminate results was significantly higher in autumn

56   (8.2%) than in spring (6.2%; p<0.0001), summer (4.8%; p<0.0001), and winter (6.2%;

57   p<0.0001). The highest proportions of indeterminate results were observed in

58   October (8.4%) and November (8.8%), the lowest in June (4.5%). Our data show that

59   significant seasonal variation occurs in the performance of QFT-GIT assays in a

60   temperate climate setting. Potential underlying mechanisms, including host and

61   environmental factors, are discussed.

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63   Word count: 150

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INTRODUCTION

Interferon-gamma release assays (IGRAs) detect interferon-gamma responses following *in vitro* stimulation of blood with mycobacterial antigens [1], and are used widely throughout the U.S. and Europe for the detection of tuberculosis (TB) infection [2]. Of the two commercially available IGRAs, QuantiFERON-TB Gold assays are more commonly used in routine clinical practice than T-SPOT.*TB* assays [2].

IGRAs have several limitations including significant proportions of indeterminate assay results in certain patient populations and suboptimal sensitivity in patients with active TB [3-5]. Additionally, numerous studies indicate that IGRAs are not particularly robust, with minor variations in sampled blood volumes, intensity of assay tube shaking and delays in sample incubation having considerable impact on interferon-gamma responses and consequently assay results [3].

Data from a recent *ex vivo* study suggest that the performance of IGRAs is also influenced by environmental temperatures to which assay tubes are exposed prior to incubation [6]. As ambient temperature is not usually controlled during sample transport in routine clinical settings, we hypothesized that IGRA performance may vary between seasons in temperate climates. This study aimed to determine whether there is seasonal variation in the performance of QuantiFERON-TB Gold In-Tube (QFT-GIT) assays.

METHODS
Materials

We retrospectively studied data from QFT-GIT assays (Cellestis/Qiagen, Carnegie, Australia) processed routinely at a large independent, accredited diagnostic service provider in London between December 2009 and May 2014. The QFT-GIT samples originated from external healthcare providers or from the in-house phlebotomy services. Samples from the former are transported to the laboratory by car or motorcycle without temperature monitoring. All samples were incubated within 16 hours, as per manufacturer's instructions. Assays were processed and interpreted according to the most current version of the manufacturer's instructions [7]. All data analyses were performed by independent researchers with no access to personal or identifiable data.

102 **Statistical analysis**

103 Categorical variables were compared using two-tailed chi square tests and

104 quantitative variables with Kruskal Wallis tests (comparisons of multiple groups) or

105 two-tailed Mann Whitney *U* tests (two-group comparisons). 95% confidence intervals

106 around proportions were calculated with the Wald method. To determine potential

107 associations between temperature and assay results, linear regression analyses

108 were performed and Spearman's rank correlation coefficients with two-tailed

109 significance values were calculated. All statistical analyses were done with Prism

110 (V7.0; GraphPad, La Jolla, U.S.).

111

112

113 **RESULTS**

114 A total of 31932 QFT-GIT assays were included in the analyses. Of these, 8044 were

115 performed in spring (March - May), 7376 in summer (June - August), 8996 in autumn

116 (September - November), and 7516 in winter (December - February).

117

118 **Analysis of positive QFT-GIT results**

119 The result was positive in 5051 (15.8%) QFT-GIT assays. The proportion of positive

120 versus other assay results (i.e. negative or indeterminate) was significantly lower in

121 autumn (n=1332; 14.8%) than in spring (n=1285; 16.0%; p=0.0366) and summer

122 (n=1290; 17.5%; p<0.0001), but similar to the proportion in winter (n=1144; 15.2%;

123 p=0.4711). The lowest proportions of positive results were observed in October

124 (12.8%), November (14.7%), and December (13.6%), while the highest proportion of

125 positive results occurred in June (18.5%) (Figure 1A).

126

127 **Analysis of indeterminate QFT-GIT results**

128 The result was indeterminate in 2052 (6.4%) QFT-GIT assays. The majority of

129 indeterminate results were due to inadequate positive control responses (n=1923;

130 93.8%) rather than high interferon-gamma concentrations in the negative control

131 samples (n=129; 6.3%). The proportion of indeterminate compared with determinate

132 (positive or negative) assay results was significantly higher in autumn (n=734; 8.2%)

133 than in spring (n=497; 6.2%; p<0.0001), summer (n=354; 4.8%; p<0.0001), and

134 winter (n=467; 6.2%; p<0.0001). The highest proportions of indeterminate results

135 were observed in October (8.4%) and November (8.8%), while the lowest proportions

136 were observed in June (4.5%), July (5.0%) and August (4.8%).

137

138   Indeterminate assay results due to inadequate positive control responses and high

139   interferon-gamma concentrations in the negative control both contributed to the high

140   proportion of indeterminate results observed in autumn (n=670 and n=64,

141   respectively, corresponding to 7.4% and 0.71% of tests during that season) (Figure

142   1B&C). Compared with autumn, the proportion of indeterminate results due to

143   inadequate positive control responses was significantly lower in spring (n=464; 5.8%;

144    $p<0.0001$), summer (n=330; 4.5%; $p<0.0001$), and winter (n=459; 6.1%; $p=0.0008$).

145   Furthermore, compared with autumn, the proportion of indeterminate test results due

146   to high interferon-gamma concentrations in the negative control was significantly

147   lower in spring (n=33; 0.41%; $p=0.0122$), summer (n=24; 0.33%; $p=0.0011$) and

148   winter (n=8; 0.11%; $p<0.0001$).

149

150   Analysis of background-corrected positive control responses (i.e. interferon-gamma

151   concentration in the positive control sample minus concentration in the negative

152   control sample) showed that median responses were significantly lower in assays

153   performed in autumn (11.94 IU/mL; IQR: 6.32 - 15.00 IU/mL) than in those performed

154   in spring (13.60 IU/mL; IQR: 8.19 - 17.54; $p<0.0001$), summer (12.62 IU/mL; IQR:

155   8.17 - 15.19 IU/mL; $p<0.0001$) and winter (13.07 IU/mL; IQR: 8.28 - 15.88 IU/mL;

156    $p<0.0001$).

157

158   **Relationship between temperature and categorical QFT-GIT results**

159   To determine whether there was a potential association between temperature and

160   categorical assay results, regression analyses using average seasonal temperatures

161   recorded in central London (St. James's Park) were performed. There was a non-

162   significant trend for a positive correlation between average temperature and the

163   proportion of positive assay results (Figure 2A). In addition, there was a statistically

164   significant, weak inverse correlation between average temperature and the

165   proportion of indeterminate assay results due to failed positive controls (r = -0.4742,

166   p = 0.0468; Figure 2B).

167

168

169   **DISCUSSION**

170   To our knowledge, this is the first study to show significant seasonal variation in the

171   performance of QFT-GIT assays, and it is one of the largest studies investigating

172   IGRAs to date.

173

174   We found that positive QFT-GIT results were significantly more common in spring

175   and summer. Furthermore, the proportion of indeterminate results was significantly

176   higher in autumn. This finding is consistent with a Swiss study that analysed 1429

177   T-SPOT.*TB* assays (which being ELISPOT-based assays differ from QFT-GIT

178   assays) performed over a single year [8]. Indeterminate T-SPOT.*TB* results were

179   significantly more common in autumn and winter combined, compared with spring

180   and summer. In addition, indeterminate results were more common in young children

181   and elderly patients, consistent with our previously reported observations in QFT-GIT

182   assays [4,9].

183

184   Seasonal variation in the performance of QFT-GIT assays may result either from host

185   factors or from environmental conditions directly impacting the assay. Significant

186   seasonal variations in the human immune system have recently been described,

187   including the cellular composition of peripheral blood and the expression of pro-

188   inflammatory genes [10]. However, environmental factors might also play a

189   substantial role, with indeterminate results increasing during colder months due to

190   samples being exposed to cooler ambient temperatures during transport. We have

191   previously shown, in a controlled laboratory setting, that QFT-GIT positive control

192   responses are significantly reduced when samples are maintained below 22°C prior

193   to incubation [6]. This hypothesis is supported by the fact that in the present study

194   the majority of indeterminate assay results were due to inadequate positive control

195   responses and were more common in autumn, when mean monthly temperatures in

196   central England ranged from 5.2°C to 15.1°C during the study period [11].

197   Furthermore, our analyses revealed that there was indeed a weak correlation

198   between average seasonal temperatures and the proportions of indeterminate test

199   results. The positive finding is more striking as the diurnal and day-to-day

200   temperature variations, which can be considerable in the U.K., were not accounted

201   for in these analyses, which may have resulted in an underestimate of the impact of

202   temperature.

203

204   Indeterminate responses due to high interferon-gamma concentrations in negative

205   control samples were also significantly more common in autumn than in other

206   seasons. Although the underlying mechanism remains uncertain, this may relate to

207   viral respiratory infections being more common in autumn than in warmer seasons,

208   as these can cause increased interferon-gamma concentrations in peripheral blood

209   [12].   Alternatively this could reflect the immune system of Europeans showing a

210   pro-inflammatory bias during autumn and winter [10].

211

212 The observation that the proportion of positive QFT-GIT results also varied between

213 seasons, with the highest proportion occurring in spring and summer, is more difficult

214 to explain. However, several studies have shown seasonal variation in the incidence

215 of active TB as well as non-tuberculous mycobacterial disease in temperate climates

216 with peaks occurring in spring and troughs in autumn [13,14].   Furthermore, in the

217 U.K. population the levels of vitamin D, which plays a crucial role in anti-

218 mycobacterial immune responses, are highest during summer months due to

219 increased sun exposure [15].   This potentially results in higher interferon-gamma

220 responses during that season, thereby leading to a greater number of positive QFT-

221 GIT assay results. The alternative explanation is that, analogous to positive control

222 responses, antigen-stimulated interferon-gamma responses are also impaired by

223 colder temperatures, resulting in more false-negative results during cooler months.

224

225 The main limitation of this study is that no clinical data were available. Therefore, the

226 number of individuals with risk factors for TB, confirmed active TB, or

227 immunocompromise, all of which can impact on individual QFT-GIT results, is

228 unknown. However, given the large size of the dataset capturing data from a 4.5-year

229 period, it is unlikely that observed differences are attributable to coincidental changes

230 in the patient population. Additionally, official population figures suggest that the

231 population of Greater London showed little variation over the study period with

232 regards to age and gender distribution [16]. A second limitation of the study is that we

233 were not able to determine the precise number of samples that were transported

234 from external healthcare providers to the diagnostic service provider (vs. samples

235 obtained in-house). However, according to information provided by the diagnostic

236 service provider, annually more than 80% of the QFT-GIT samples originate from

237 external providers, confirming that the majority of samples have been exposed to

238 ambient temperatures.

239

240 In conclusion, there is significant seasonal variation in the performance of QFT-GIT

241 assays in a temperate climate setting. Both environmental and host factors are

242 potentially causally implicated in the high proportion of indeterminate results during

243 autumn and winter. Further research is required to determine whether temperature

244 control during sample transport can improve assay performance during colder

245 periods of the year, generating more reliable results for patient care.

257

258 **Contributors:** Study concept: MT and SM-J. Data analyses: MT, NC, VC, PE and
259 SM-J. Data interpretation: MT, NC, VC, CF-T, NK, KF, SM, PE and SM-J. Drafting of
260 the manuscript: MT, NC, PE and SM-J. All authors critically read, commented on,
261 and approved the final version of the manuscript.

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263 **Ethical considerations:** Under current U.K. health research ethics regulations,
264 research limited to secondary use of information previously collected in the course of
265 normal care without intention to use it for research does not require research ethics
266 committee review, if patients or service users are not identifiable to the research
267 team.

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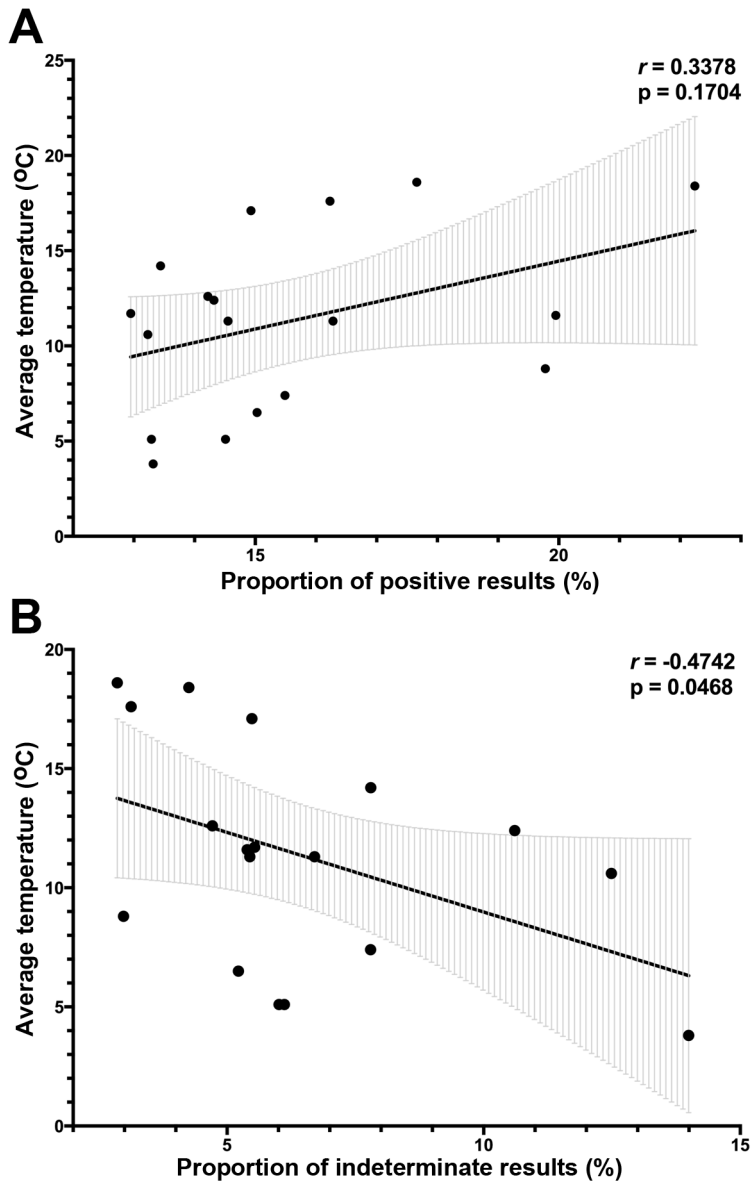318   age-tables (accessed 3 March 2018).
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321   **Figure 1.** Proportions of (A) positive results, (B) indeterminate results due to

322   insufficient interferon-gamma responses in the positive control sample, and (C)

323   indeterminate results due to high interferon-gamma concentrations in the negative

324   control sample in QuantiFERON-TB Gold In-Tube assays according to month and

325   season. The whiskers represent the 95% confidence intervals. The p-values shown

326   were calculated with Kruskal Wallis tests.

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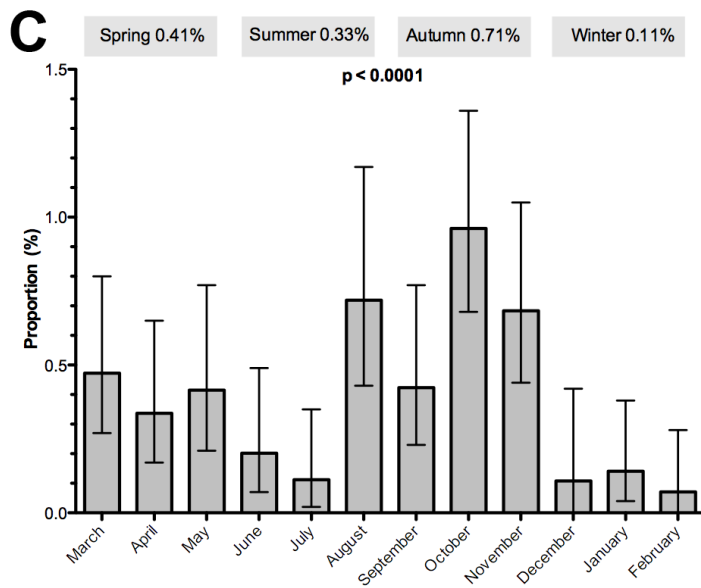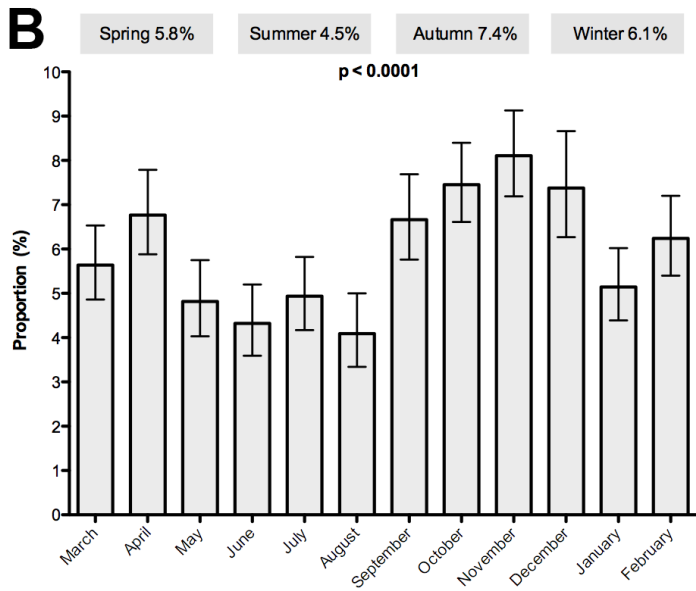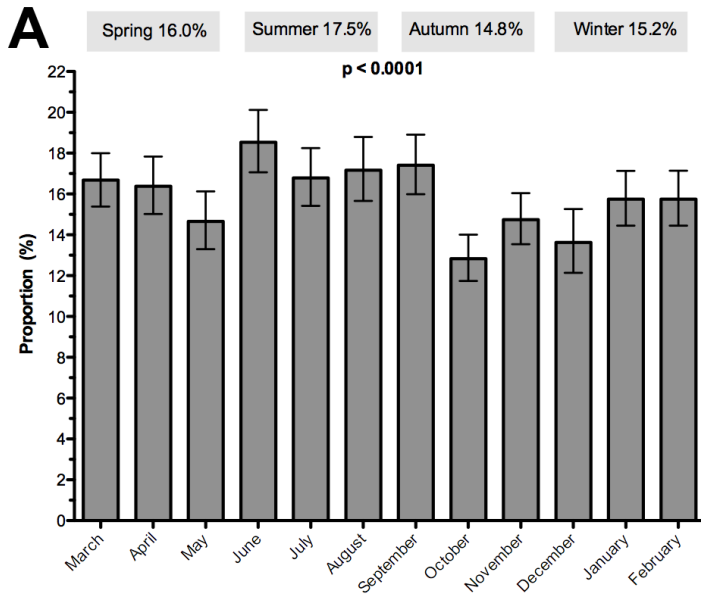
330 **Figure 2**. Relationship between average seasonal temperatures and categorical
331 QuantiFERON-TB Gold In-Tube assay results. (A) Positive assay results; (B):
332 indeterminate assay results due to failed positive controls. The graphs show the
333 regression line (black) with 95% confidence intervals (grey), the Spearman's rank
334 correlation coefficient (r) and the corresponding p-value (p).
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