Low oxygen arrests *Babesia duncani* schizonts and leads to increased drug susceptibilities in hamster erythrocytes

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Abstract

We studied the effect of oxygen concentrations on the in vitro growth and drug susceptibility of *Babesia duncani*. We found that the growth of *B. duncani* required high level oxygen and the culture condition at ambient aerobic condition (21% O₂) was optimal. Compared with ambient air, our results further showed that low oxygen (6-16%) could arrest *B. duncani* schizonts and lead to high susceptibilities to antiparasitic drugs atovaquone, pyrimethamine, quinine, and chloroquine at certain concentrations in vitro. Drug susceptibilities of other *Babesia* spp impacted by O₂ levels need to be studied in the future, and this study indicates that culturing conditions of *Babesia* spp should be considered and reestablished for generating more comparable and reliable results in drug research in the future.

Keywords: *Babesia duncani*, oxygen, drug susceptibility

24 Graphical abstract

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28 Highlights

- 29 • The in vitro growth of *B. duncani* requires high level oxygen and the culture
- 30 condition of 5% CO₂ ambient air is optimal
- 31 • Low level oxygen results *B. duncani* in low growth rate and high schizont ratio
- 32 in vitro
- 33 • Compared with 5% CO₂ ambient air, in vitro drug susceptibilities of *B. duncani*
- 34 can be significantly increased when cultured in microaerobic condition

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37 Babesiosis is one of the most important tick-borne infectious diseases in Europe and
38 North America and has been added to the list of nationally notifiable disease by the
39 Centers for Disease Control and Prevention (CDC) of the United States . The clinical
40 symptoms of babesiosis patients range from flu-like mild to malaria-like severe
41 manifestations and can even cause death in elderly and immunocompromised patients.
42 However, the current therapeutic regimen to human babesiosis is suboptimal with
43 significant side effects and occasionally recrudescence (Kjemtrup and Conrad, 2000).

44 For development of more effective treatments, continuous in vitro culture of
45 *Babesia* spp is one of the most critical techniques to explore active drugs. In the past
46 decade, dozens of promising anti-babesia compounds have been identified with this
47 technique. The first attempt to establish in vitro cultivation of *Babesia* spp in any real
48 sense was in 1980, when Timms cultivated *B. bovis*, *B. bigemina*, and *B. rodhaini* in
49 vitro up to 96 h using the candle jar technique which mimicked the in vitro culture of
50 *Plasmodium falciparum* by Trager and Janson (Jensen and Trager, 1977; Timms, 1980).
51 Subsequently, Levy and Ristic successfully carried out continuous cultivation of *B.*
52 *bovis* in a stationary microaerophilic phase where oxygen had decreased to the level that
53 could darken red blood cells laid on plate bottom (Levy and Ristic, 1980). In 1985,
54 Vega *et al.* further demonstrated that high oxygen (5.6% - normal air) is detrimental to
55 in vitro growth of *B. bigemina* (Vega et al., 1985). Thus, it seems that *Babesia* spp are
56 microaerophilic organisms like *P. falciparum*. However, the in vitro cultivations of two
57 important human babesiosis causative agents *B. divergens* and *B. duncani* were set in
58 different O₂ levels in some studies for drug activity testing or drug screening (Abraham
59 et al., 2018; Brasseur et al., 1998; Nehrbass-Stuedli et al., 2011; Zhang et al., 2020),
60 which have could lead to divergent results in anti-malarial drug studies (Branco et al.,
61 2018; Duffy and Avery, 2018). Thus, two interesting questions are raised here: first,

62 does *B. duncani* grow well in low O₂; second, is O₂ level a factor that affects the anti-
63 babesia drug activity in vitro?

64 To address these questions, here we studied the effects of O₂ levels on the
65 growth and drug tolerance of *B. duncani* (WA1 strain), the latest human babesiosis
66 pathogen to be identified. In continuous culture model in hamster erythrocytes
67 cultivated in 5% CO₂ ambient air with humidity (Zhang et al., 2020), we found *B.*
68 *duncani* schizont was the major morphology in old cultures (5-6 culture days, 0.5% of
69 initial parasitemia) and this morphology rarely appeared in young cultures (Figure 1A).
70 These schizonts appearing as budding four daughter merozoites in erythrocytes and
71 displayed several different scattered distributions (Figure 1, B1-B6). Considering
72 culture medium changed daily and erythrocytes turned dark in old cultures, we
73 hypothesize that low oxygen environment resulting from rapid oxygen consumption by
74 parasites in high parasitemia is related to the increasing percentage of schizonts. To
75 confirm this, when parasitemia reached to 1.5-2% cultivated in 5% CO₂ atmosphere, we
76 split the *B. duncani* cultures into anaerobic, microaerobic, and aerobic conditions
77 without other culturing condition change to evaluate the effects of oxygen
78 concentrations on parasite growth and morphologic development. In this study,
79 anaerobic (0.7-1% O₂) and microaerobic (6-16% O₂) conditions were generated by BD
80 GasPak EZ container systems with CampyPak container system sachets and anaerobe
81 container system sachets, respectively, and 5% CO₂ atmosphere was viewed as aerobic
82 condition (21% O₂, approximately). Even though the final CO₂ concentrations in BD
83 GasPak EZ container systems would be various from 2% to 13% according to the
84 manufactory's instructions, our results indicated that the rate of growth and
85 morphologic development of *B. duncani* did not show significant difference among 2, 5,
86 and 13% CO₂ atmosphere air the cultures incubated in, but *B. duncani* did not grow in

atmosphere air deprived CO₂ (data not shown). Culture medium covered erythrocytes replaced every day with fresh medium which had been co-incubated in respective culture environments to minimize the impact of the fluctuation of nutrition and dissolving oxygen.

Our results showed that the growth of *B. duncani* incubated in microaerophilic condition reached to peak parasitemia of approximate 16% at day 5. As a comparison, the parasitemia of cultures incubated in aerobic condition could finally reach to approximate 40% at day 5 before erythrocyte lysis, and it grew faster than that in microaerophilic condition after 1 day (Figure 2). Growth of *B. duncani* stopped when incubated in anaerobic condition, and no infected erythrocyte was observed starting at day 3 (Figure 2). This indicates that *B. duncani* is an oxygen preferred organism, and the growth of *B. duncani* requires more oxygen than only 2-5% O₂ employed in previous studies which may not be the optimal in vitro growth condition for *B. duncani* (Abraham et al., 2018; McCormack et al., 2019). It can partly explain why the *B. duncani* cultures obtained in ambient air was able to reach to much higher parasitemia than reported parasitemias, i.e., 40% in our study vs 20% or less previously (Abraham et al., 2018; McCormack et al., 2019). Of note, the pericellular O₂ around the parasites and erythrocytes laid on plate bottom must be lower than environmental O₂ due to the culture medium with depth of 0.7cm in our culturing system which acted as an oxygen barrier.

In the human body, mature *P. falciparum* parasites (trophozoites and schizonts) exhibit a preference for the venules where relatively high O₂ levels are present (Milner et al., 2015). Research on the effect of oxygen on the growth of *Babesia* spp has not been reported in vitro or in vivo. In contrast, our in vitro results showed *B. duncani* schizonts were more likely arrested in anaerobic and microaerobic conditions than

112 ambient oxygen rich condition (Figure 2). Specifically, even though *B. duncani* did not
 113 grow in anaerobic container (0.7-1% O₂), the ratio of schizonts in infected erythrocytes
 114 soared immediately at the beginning and could finally reach to nearly 70% schizonts
 115 before all parasites died out (Figure 2). The ratios of schizonts were increasing along
 116 with increasing parasitemia and oxygen consumption in both microaerobic and aerobic
 117 conditions, but the ratios of schizonts cultured in microaerobic condition were higher
 118 than that cultured in aerobic condition at each day and entered plateau one day earlier.
 119 This indicates that low O₂ is able to extend the duration of *B. duncani* schizonts in vitro
 120 or prevent schizonts from lysing erythrocytes to generate merozoites, which could also
 121 happen in late culture phase. In contrast, Briolant et al. found that *P. falciparum* clones
 122 3D7 and W2 had higher percentages of schizonts when inoculated in 21% O₂ than that
 123 inoculated in 5% O₂, even though the growth of these two *P. falciparum* clones did not
 124 show significant difference in 21% and 5% O₂ (Briolant et al., 2007).

125 Slight variations of in vitro culture environments have demonstrated significant
 126 impact on the conclusions of *P. falciparum* drug resistance (Duffy and Avery, 2018). In
 127 our study, we found that *B. duncani* cultured in microaerobic condition showed
 128 significantly higher susceptibility to four anti-parasite drugs atovaquone,
 129 pyrimethamine, quinine, and chloroquine at 0.25-50 µM than that cultured in aerobic
 130 condition, except that atovaquone had almost 100% inhibitory effects against *B.*
 131 *duncani* at 2.5, 25, and 50 µM in both aerobic and microaerobic conditions (Figure 3).
 132 The most variation of inhibitory effects occurred in chloroquine exposure, which had
 133 65-100% inhibitory effect on *B. duncani* at 0.25 and 2.5 µM in microaerobic condition,
 134 but the effect was only 9-34% at the same drug concentrations in aerobic condition
 135 (Figure 3). As mentioned above, given *P. falciparum* 3D7 and W2 have the opposite
 136 manifestation of ratios of schizonts in different O₂ levels compared to *B. duncani* in this

study, it is not surprising that *P. falciparum* 3D7 and W2 were more susceptible to chloroquine in aerobic condition than that in microaerobic condition (Briolant et al., 2007), which is dissimilar to the chloroquine susceptibility of *B. duncani* as shown in our study. We speculated that these different consequences could result from the same reason that schizonts are more susceptible to chloroquine due to chloroquine accumulation might be much greater in schizont stage than in ring stage (Yayon et al., 1983). Besides, some compounds have shown various activities against highly synchronous age defined *P. falciparum* parasites (Duffy and Avery, 2017), however, to date there is no way to study drug susceptibility of *Babesia* spp at a certain life cycle stage due to the lack of effective synchronization of *Babesia* spp. Drug susceptibilities of other *Babesia* spp impacted by O₂ levels need to be studied in the future, and culturing conditions of *Babesia* spp should be reestablished for generating more comparable and reliable results in drug research and the relevant conditions that reflect in vivo drug treatment effect should be determined to identify more effective drugs.

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206 Figure 1. (A) *B. duncani*-infected hamster erythrocytes observed at 1,000×
 207 magnification in young (2 days old; left) and old (5-6 days old; right) cultures with
 208 parasitemia of approximate 1.5% and 40%, respectively. The growth of parasites started
 209 from 0.5% parasitemia; (B) *B. duncani* schizonts appearing as budding four daughter
 210 merozoites in erythrocytes displayed several different scattered distributions. B1,
 211 Maltese Cross tetrad; B2, four daughter merozoites connected in pairs; B3, three
 212 daughter merozoites assembled with one in isolation; B4, the moment of matured
 213 merozoites bursting erythrocyte; B5, two schizonts in one erythrocyte; B6, a schizont
 214 accompanied with ring form trophozoite;

215 Figure 2. Growth (left) and percentage of schizonts (right) of *B. duncani* cultures
 216 incubated in aerobic, microaerobic, and anaerobic environments;

217 Figure 3. *B. duncani* incubated in aerobic and microaerobic conditions with a starting
 218 1.5% parasitemia and 2.5% hematocrit (2 days old culture) treated with atovaquone,
 219 pyrimethamine, quinine, and chloroquine at 0.25, 2.5, 25, 50 µM after 3 days exposure.

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221 Figure 1

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224 Figure 2

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