RESEARCH ARTICLE

Antiplasmodial Activity of Artemether-Lumefantrine-Tinidazole on *Plasmodium Berghei* Infected Mice

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ABSTRACT

**Introduction:** The impact of malaria scourge has been characterized by daunting challenges including antimalarial drug resistance. This necessitates the search for newer antimalaria drugs using approaches including drug repurposing. This study assessed whether Tinidazole (T) can be repurposed as antimalaria in combination with artemether/lumefantrine (A/L) in *Plasmodium berghei* infected mice.

**Materials and Methods:** *Plasmodium berghei* infected mice were grouped and orally treated with A/L (2.3/13.7 mg/kg), T (28.6 mg/kg), and A/L/T daily in curative, suppressive and prophylactic studies. The negative control (NC) and positive control (MC) were orally treated with 0.9% normal saline (0.2mL) and chloroquine (CQ) (10mg/kg) daily for 4 days, respectively. After drug administration, blood samples were collected and evaluated for parasitemia level, lipid and hematological parameters.

**Results:** Significant decreases in parasitemia levels in the curative, suppressive and prophylactic groups were observed in mice treated with T (28.6 mg/kg) (p<0.05), A/L (2.3/13.7 mg/kg) (p<0.01) and A/L/T (p<0.001) when compared to negative control. Mean survival times were significantly increased at T (28.6 mg/kg) (p<0.05), A/L (2.3/13.7mg/kg) (p<0.01) and A/L/T (p<0.001) when compared to negative control. Red blood cells, hemoglobin, packed cell volume, high density lipoprotein, cholesterol levels were significantly (p<0.001) increased whereas white blood cells, total cholesterol, triglyceride and low density lipoprotein cholesterol levels were significantly decreased at T (28.6 mg/kg) (p<0.05), A/L (2.3/13.7mg/kg) (p<0.01) and A/L/T (p<0.001) when compared to negative control. The antiplasmodial effect of A/L/T differ significantly (p<0.05) when compared to positive control.

**Conclusion:** This study recommends the repurposing of tinidazole in combination with artemether/lumefantrine for malaria treatment and further studies in humans.

**KEYWORDS:** Tinidazole; Artemeter/Lumefantrine; Antiplasmodial; *P. berghei*; Mice.

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INTRODUCTION

Malaria is one of the protozoan parasites that have caused significant health challenge to man. It is primarily endemic in sub-tropical and tropical parts of the world. Malaria affects about 3.3 billion people and causes 0.6–1.1 million mortality annually [1]. African countries have the heaviest malaria burden; globally it records 90% of all malaria infections and 92% of malaria associated mortality which significantly impairs economic growth and development [2]. Most deaths associated with malaria are caused by *Plasmodium falciparum* (*P. falciparum*) and occurred in sub-Saharan Africa whereas malaria death in Asia Pacific region and South America is caused by *P. vivax* [3]. Despite measures to curtail malaria infection especially in Africa its impact surges annually. Unfortunately, challenges such as drug resistance, cost and decreased accessibility, affects currently available antimalarial drugs including artemisinin based combinations, arguably the current hope and primary line of defense against malaria scourge [4,5]. The ongoing search to for newer and effective antimalarial drugs that will overcome multi-drug resistant parasites has instigated researchers to invest in various drug discovery and development methods. Drug repurposing is one of the methods of drug discovery that has played a significant
role in malaria therapy to date and has attracted renewed interest in recent times for the search for newer antimalarial drugs [6]. The repositioning, or repurposing of already established drugs used for other indications will help discover newer therapies for other diseases. This approach has cutting edge advantages including cost reduction, decreased time and reduced delay in drug discovery and development. It has become an important research method used by the pharmaceutical industry with notable achievement reported in 2004 where 40% of United States Food and Drug Administration (FDA) registered drugs were repurposed [7,8].

Tinidazole (T) (5-nitroimidazole), a widely used drug for the treatment of giardiasis and amoebiasis has proven safety profile [9,10]. Despite, its current indications, it has exhibited potential antimalarial activity as captured in emerging studies. Prophylaxis testing of Tinidazole in chick model with P. gallinaceum increased survival time from 4 days to 9.5 days [11]. It cured liver stage of infection and delayed relapse in ‘Rhesus’ macaques (Macaca mulatta) infected with relapsing strain of P. cynomolgi and eliminated blood stage of infection in combination with chloroquine (CQ) [10]. In humans with P. vivax, tinidazole monotherapy cleared blood stage infection within 96 hours with no relapse [9]. Experimentally, due to increased antimalarial activity when coupled with CQ, this study assessed whether T can be repurposed for malaria treatment in combination with artemether/lumefantrine (A/L) in P. berghei infected mice.

MATERIALS AND METHODS

Drugs
Chloroquine (CQ) (Evans Medical Nigeria Plc), tinidazole (T) (Norvatis) and artemether/lumefantrine (A/L) (IPAC Laboratory, India) were used for this study. CQ (10mg/kg) [12], A/L (2.3/13.7 mg/kg) [13] and T (28.6 mg/kg) [14] were used.

Experimental Animals
Swiss albino mice (25-30g) were used for this study. The mice were purchased from the animal breeding unit of the Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Rivers State, Nigeria. The mice were kept in plastic cages of 5/group at a temperature of 28.0 ± 2.0°C and a 12 hour light/dark cycle. The mice were acclimatized for 2 weeks prior to the experiment with free access to diet and water. The mice were handled according to the directive (2010/63/EU) of the European Union Parliament and the Council on the handling of animals for scientific purposes.

Malaria Parasite
A CQ sensitive strain of P. berghei supplied by Nigerian Institute of Medical Research, Yaba, Lagos was used for this study. The P. berghei was maintained by serial blood passage from mouse to mouse every 5-7 days. Blood samples were obtained from donor parasitized mice with a parasitemia of 20-30% into heparinized tubes and diluted with 0.9% normal saline. The mice were infected intraperitoneally (i.p) with the diluted blood sample (0.2 mL) containing 1x10^7 parasitized erythrocytes. Daily parasitemia levels were monitored using microscopic examination of Giemsa stained thin blood smears. Parasitemia was calculated using the relationship below.

% Parasitemia = \( \frac{\text{Total number of parasitized RBC}}{\text{Total number of RBC}} \times 100 \)

Evaluation of Suppressive Antiplasmodial Activity
Suppressive antiplasmodial test was evaluated as described by Knight and Peters (1980) [15]. Twenty five mice were inoculated with 1 X 10^7 P. berghei parasitized erythrocytes i.p and randomized into 5 groups (A1-A5) of n=5. After two hours, the mice were treated as follows: Group A1 (Negative control) (NC) was treated with normal saline (0.2mL), group A2 (Positive control) was treated with CQ (10mg/kg) whereas group A3 was treated with T (28.6 mg/kg/day) for 4 days. Group A4 was treated with A/L (2.3/13.7 mg/kg) whereas group A5 was treated with A/L/T for 4 days. On the 5th Day, blood samples were collected and thin films produced on microscope slides. The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 10 min and examined for parasitemia using a microscope. Parasitemia levels were ascertained by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. Average percentage inhibitions were calculated using the formula below:

\[ \frac{\text{Mean parasitemia of negative control} - \text{Mean parasitemia of treated groups}}{\text{Mean parasitemia of negative control}} \times 100 \]

Evaluation of Curative Antiplasmodial Activity
Curative antiplasmodial activity was evaluated as described by Ryley and Peter (1970) [16]. Thirty mice were randomized into 6 groups (B1-B6) of n=5. Group B1 severed as normal control (MC) whereas the experimental groups B2-B6 were inoculated with 1 X 10^7 P. berghei parasitized erythrocytes i.p. Seventy two hours later (Day 3), the groups were treated as follows: Group B1 (normal control) and group B1 (Negative control) were treated with normal saline (0.2mL) whereas group B3 (Positive control) was treated with CQ (10mg/kg) daily for 4 days. Group B4 was treated with T (28.6 mg/kg/day), group B5 was treated with A/L (2.3/13.7 mg/kg) whereas group B6 was treated with A/L/T daily for 4 days. Thin films were made from collected blood samples, fixed in methanol and stained with 10% Giemsa at pH 7.2 for 10 min. Parasitemia was determined microscopically and percent inhibitions were then calculated as described above.

Evaluation of Prophylactic Antiplasmodial Activity
Prophylactic antiplasmodial activity was determined according to Peters (1967) [17]. Twenty five mice, parasitized with 1 X 10^7 P. berghei were randomized into 5 groups (C1-C5) of n=5. The mice were pre-treated as follows: Group C1 (Negative control) was treated with normal saline (0.2mL), group C2 (Positive control) was treated with CQ (10mg/kg) whereas group C3 was treated with T (28.6 mg/kg/day) for 4 days. Group C4 was treated with A/L (2.3/13.7 mg/kg) whereas group C5 was treated
with A/L/T for 4 days. Thereafter, the mice were inoculated with 1×10⁶ P. berghei infected erythrocytes i.p and treatment continued. On day 1, 3, 5 and 7 percentage parasitemia levels were determined. Percentage inhibitions were calculated on day 7 as described above.

**Determination of mean survival time**

The mice were observed daily for mortality. The number of days from the beginning of infection to death for each mouse in the control and experimental groups were recorded. Mean survival time (MST) was calculated using the formula below.

\[
\text{MST} = \frac{\text{Sum of survival time (days) of all the mice in the group}}{\text{Total number of mice in that group}}
\]

**Data Analysis**

GraphPad prism 6.0 statistical software was used for data analysis and data are expressed as Mean±SEM. Significant difference among groups were determined using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. A p value less than 0.05; 0.01 and 0.001 was considered significant.

**RESULTS**

**Suppression and Curative Tests**

In the suppression and curative tests, treatment with individual doses of T and A/L produced significant reductions in percentage parasitemia levels at p<0.05 and p<0.01 respectively when compared to negative control. On the hand, most significant (p<0.001) reductions in percentage parasitamia levels were observed in rats treated with A/L/T when compared to negative control. The reduction in percentage parasitemia level in mice treated with A/L/T differ at p<0.05 when compared to positive control (CQ) (Tables 1 and 2). In the curative test, T, A/L and A/L/T produced percentage parasitemia inhibitions of 60.3%, 70.8% and 99.1% respectively compared to 71.0% produced by CQ. In the suppressive and curative tests, treatment with individual doses of T and A/L significantly increased MST at p<0.05 and p<0.01 respectively when compared to negative control (Tables 1 and 2). Interestingly, treatment with A/L/T produced most significant decreases in WBC, TG, CHOL, and LDL-C levels when compared to normal control (Tables 4 and 5). However, treatment with individual dose of T and A/L significantly increased RBC, HB, PCV and HDL-C levels and significantly decreased WBC, TG, CHOL, and LDL-C levels at p<0.05 and p<0.01 respectively when compared to negative control (Tables 4 and 5).

**Effects on hematological and lipid parameters**

The negative control shows significant (p<0.001) decreases in RBC, HB, PCV and HDL-C levels with significant (p<0.001) increases in WBC, TG, CHOL, and LDL-C levels when compared to normal control (Tables 4 and 5). Interestingly, treatment with A/L/T produced most significant increases in RBC, HB, PCV and HDL-C levels with most significant decreases in WBC, TG, CHOL, and LDL-C levels at p<0.001 when compared to negative control. The observed effects produced by A/L/T on RBC, HB, PCV, HDL-C, WBC, TG, CHOL, and LDL-C levels differ from CQ at p<0.05 (Tables 4 and 5).

**Table 1: Curative antiplasmodial effect of artemether-lumefantrine-tinidazole on Plasmodium berghei infected mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Parasitemia (%)</th>
<th>Inhibition (%)</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>35.9±4.02</td>
<td>-</td>
<td>10.8±1.32</td>
</tr>
<tr>
<td>CQ</td>
<td>10.3±0.06b</td>
<td>71.0</td>
<td>27.3±3.37b</td>
</tr>
<tr>
<td>T</td>
<td>14.3±0.73b</td>
<td>60.3</td>
<td>20.8±2.44</td>
</tr>
<tr>
<td>A/L</td>
<td>10.7±0.37c</td>
<td>70.8</td>
<td>25.0±2.20</td>
</tr>
<tr>
<td>A/L/T</td>
<td>0.27±0.01c</td>
<td>99.1</td>
<td>30.5±2.71b</td>
</tr>
</tbody>
</table>

With the following referring to: NC: Negative control ; CQ: Chloroquine ; T: Tinidazole ; A/L : Artemether/lumefantrine ; A/L/T: Artemether/lumefantrine/tinidazole ; MST: Mean survival time ; n=5. Values are expressed as Mn±SEM, a p<0.01 when compared to NC, b p<0.05 when compared to NC, c p<0.01 when compared to NC, * p<0.001 when compared to NC, * p<0.05 when compared to CQ.

**Table 2: Suppressive Antiplasmodial Effect of Artemether-lumefantrine-tinidazole on Plasmodium berghei Infected Mice.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Parasitemia (%)</th>
<th>Inhibition (%)</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>24.9±2.02</td>
<td>-</td>
<td>6.0±0.20</td>
</tr>
<tr>
<td>CQ</td>
<td>0.59±0.27a</td>
<td>97.6%</td>
<td>29.7±1.33a</td>
</tr>
<tr>
<td>T</td>
<td>8.00±0.26b</td>
<td>68.0%</td>
<td>21.5±1.42b</td>
</tr>
<tr>
<td>A/L</td>
<td>3.38±0.08c</td>
<td>86.4%</td>
<td>27.7±2.51c</td>
</tr>
<tr>
<td>A/L/T</td>
<td>0.03±0.02c</td>
<td>99.9%</td>
<td>34.1±3.41c</td>
</tr>
</tbody>
</table>

With the following referring to: NC: Negative control ; CQ: Chloroquine ; T: Tinidazole, A/L: Artemether/lumefantrine ; A/L/T: Artemether/lumefantrine/tinidazole ; MST: Mean survival time ; n=5. Values are expressed as Mn±SEM, a p<0.01 when compared to NC, b p<0.05 when compared to NC, * p<0.01 when compared to NC, * p<0.001 when compared to NC, * p<0.05 when compared to CQ.
In this study, the parasitological cure rate was significantly higher in the A/L/T group compared to CQ and NC (p<0.001). The cure rate was also higher in the A/L/T group compared to NC (p<0.01). The A/L/T group showed a significant increase in hemoglobin levels compared to NC (p<0.05). The A/L/T group also showed a significant decrease in white blood cell count compared to NC (p<0.001). The A/L/T group showed a significant increase in total cholesterol levels compared to NC (p<0.01) and MC (p<0.001). The A/L/T group showed a significant decrease in triglyceride levels compared to NC (p<0.05) and MC (p<0.001). The A/L/T group showed a significant increase in mean survival time compared to NC (p<0.05) and MC (p<0.001).

Table 3: Prophylactic Antiplasmodial Effect of Artemether-lumefantrine-tinidazole on Plasmodium berghei Infected Mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>DAY 4</th>
<th>DAY 5</th>
<th>DAY 6</th>
<th>DAY 7</th>
<th>inhibition (%)</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>11.5±1.2</td>
<td>18.2±1.88</td>
<td>23.3±1.58</td>
<td>24.9±2.02</td>
<td>10.7±0.22</td>
<td></td>
</tr>
<tr>
<td>CQ</td>
<td>10.4±0.35</td>
<td>10.05±0.33</td>
<td>8.48±0.52</td>
<td>6.82±0.27a</td>
<td>89.6</td>
<td>30.0±3.15a</td>
</tr>
<tr>
<td>T</td>
<td>10.8±0.92b</td>
<td>12.8±0.32b</td>
<td>9.05±0.28b</td>
<td>7.92±0.40b</td>
<td>68.8</td>
<td>23.5±2.77b</td>
</tr>
<tr>
<td>A/L</td>
<td>9.24±0.26c</td>
<td>6.82±0.23c</td>
<td>1.46±0.15c</td>
<td>2.78±0.08c</td>
<td>88.8</td>
<td>27.7±3.20c</td>
</tr>
<tr>
<td>A/L/T</td>
<td>7.29±0.82bc</td>
<td>4.82±0.24bc</td>
<td>1.12±0.05bc</td>
<td>0.03±0.00c</td>
<td>99.9</td>
<td>36.8±3.71bc</td>
</tr>
</tbody>
</table>

With the following referring to: NC: Negative control; CQ: Chloroquine; T: Tinidazole; A/L: Artemether/lumefantrine; A/L/T: Artemether/lumefantrine/tinidazole; MST: Mean Survival Time; n=5.

Values are expressed as MaSEM. *p<0.001 when compared to NC. **p=0.01 when compared to NC. \*p=0.05 when compared to NC. d p<0.01 when compared to MC. e p<0.05 when compared to MC. e p<0.001 when compared to MC. f p<0.05 when compared to MC.

Table 4: Effect of Artemether-lumefantrine-tinidazole on Lipid Profile of Plasmodium berghei Infected Mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>TG mg/dL</th>
<th>CHOL mg/dL</th>
<th>HDL-C mg/dL</th>
<th>LDL mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>112.0±7.03</td>
<td>160.5±2.46</td>
<td>55.4±1.49</td>
<td>2.14±0.05</td>
</tr>
<tr>
<td>CQ</td>
<td>328.6±18.9</td>
<td>467.9±17.6</td>
<td>20.2±1.36</td>
<td>6.18±0.5a</td>
</tr>
<tr>
<td>T</td>
<td>193.0±5.87</td>
<td>234.1±2.40</td>
<td>41.4±0.85</td>
<td>3.50±0.16</td>
</tr>
<tr>
<td>A/L</td>
<td>203.4±3.03</td>
<td>327.4±8.24</td>
<td>30.4±0.58</td>
<td>4.20±0.08</td>
</tr>
<tr>
<td>A/L/T</td>
<td>203.7±4.87</td>
<td>224.3±10.0</td>
<td>40.1±1.51</td>
<td>3.28±0.15</td>
</tr>
</tbody>
</table>

With the following referring to: MC: Normal control; NC: Negative control; CQ: Chloroquine; T: Tinidazole; A/L: Artemether/lumefantrine; A/L/T: Artemether/lumefantrine/tinidazole; TG: Triglyceride; CHOL: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; n=5.

Values are expressed as MaSEM. *p<0.001 when compared to MC. **p=0.01 when compared to MC. \*p=0.05 when compared to MC. d p<0.01 when compared to NC. e p<0.05 when compared to NC. e p<0.001 when compared to NC. f p<0.05 when compared to NC.

Table 5: Effect of Artemether-lumefantrine-tinidazole on Hematological Parameters of Plasmodium berghei Infected Mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>RBC x10⁶</th>
<th>WBC x10⁶</th>
<th>PCV %</th>
<th>HB g/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>5.7±0.18</td>
<td>6.1±0.18</td>
<td>63.2±6.74</td>
<td>17.0±1.19</td>
</tr>
<tr>
<td>CQ</td>
<td>3.2±0.13</td>
<td>11.2±1.32</td>
<td>23.8±3.86</td>
<td>8.9±0.61</td>
</tr>
<tr>
<td>T</td>
<td>5.42±0.11b</td>
<td>6.00±0.10a</td>
<td>50.6±5.74</td>
<td>14.9±1.53</td>
</tr>
<tr>
<td>A/L</td>
<td>3.7±0.09e</td>
<td>8.04±0.30b</td>
<td>37.2±3.66</td>
<td>11.8±0.45</td>
</tr>
<tr>
<td>A/L/T</td>
<td>5.08±0.13d</td>
<td>5.96±0.31c</td>
<td>46.6±4.21e</td>
<td>14.6±0.22</td>
</tr>
</tbody>
</table>

With the following referring to : MC: Normal control; NC: Negative control; CQ: Chloroquine; T: Tinidazole; A/L: Artemether/lumefantrine; A/L/T: Artemether/lumefantrine/tinidazole; RBC: Red blood cells; WBC: White blood cells; PCV: Hemoglobin; HPV: Hemoglobin percent; HB: Hemoglobin; a p<0.001 when compared to NC. b p<0.01 when compared to NC. c p<0.05 when compared to NC. d p<0.001 when compared to NC. e p<0.05 when compared to NC.

DISCUSSION

Drug repurposing, redirecting, repositioning, or reprofiling is a discovery process used to identify safe compound or drugs with proven clinical efficacy for the treatment of new or existing diseases. As repurposed drugs are already approved for used clinically, this discovery process causes significant time and cost savings [6]. Drug repurposing includes leveraging on the usefulness of existing drugs by discovering new formulations with varying strengths, combinations and dosing regimens [18]. The search for newer antimalarial drugs through repurposing of existing drugs indicates for other diseases has taken the centre stage in malaria research. This is due to surge in malaria scourge associated with daunting challenges including resistance to current antimalaria drugs [19]. T is a drug primarily indicated for amoebiasis and giardiasis, but has experimentally shown potential antimalarial property. This study assessed whether it can be repurposed as an antimalaria drug in combination with A/L using a mouse model of Plasmodium berghei-induced malaria. Over the years, curative, suppressive, prophylactic tests have been used and validated as standard experimental procedures for the evaluation of antimalaria drug candidates [20]. In this study, curative, suppressive, prophylactic antimalarial assessments of A/L/T showed best decreases in percentage parasitemia levels and increases in percentage parasitemia inhibition than individual doses of T, A/L, and CQ. Malaria is a severe public health problem worldwide. In developing countries, it is a leading cause of mortality with children and pregnant women most affected [2]. One of the primary goals of antimalaria therapy is absolute reduction in mortality rate or the achievement of zero mortality. MST is experimentally used to assess the abilities of antimalarial drug candidates to reduce mortality in animal models of Plasmodium parasite-induced malaria [20]. In this study, curative, suppressive, prophylactic antimalarial assessments of A/L/T showed increases in MST better than Q. Malaria-induced anemia which is common in malaria endemic regions is a significant public health concern. Malaria-induced anemia is characterized by hemolysis of infected and uninfected erythrocytes and bone marrow dyserythropoiesis [21]. In this study, the parasitized untreated mice (Negative control) showed signs of anemia characterized by decreased RBC, HB and PCV levels with increased WBC level. However, decrease in anemia was
observed in rats treated with individual doses of A/L/T characterized by increased RBC, HB and PCV levels with decreased WBC levels better than individual doses of T and A/L. The effects produced by A/L/T on RBC, HB, PCV and WBC levels were also better than CQ. Emerging studies showed the reliance of malaria parasites on cholesterol and phospholipids for survival in their host [22]. Erythrocytic membrane and circulating HDL particles are the sources of cholesterol, whereas erythrocyctic membrane is the source of phospholipids for malaria parasites [23]. Some population-based studies reported alterations in serum TG, CHOL, HDL, and LDL in malaria especially in endemic regions caused by P. falciparum [24]. The current study observed increased TG, CHOL and LDL-C levels with decreased HDL-C level in negative control. Interestingly, treatment with individual doses of T and A/L decreased TG, CHOL and LDL-C levels and increased HDL-C levels. However, effects on the aforementioned lipid parameters were significant in rats treated with A/L/T.

CONCLUSION
This study shows that A/L/T may be an effective treatment for malaria. We recommend further studies in humans.

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AUTHORS’ CONTRIBUTIONS
The participation of each author corresponds to the criteria of authorship and contributorship emphasized in the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly work in Medical Journals of the International Committee of Medical Journal Editors. Indeed, all the authors have actively participated in the redaction, the revision of the manuscript, and provided approval for this final revised version.

COMPETING INTERESTS
The authors declare no competing interests with this case.

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REFERENCES