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RESEARCH

Susceptibility patterns of *Bithynia siamensis siamensis* and *Bithynia funiculata* to *Opisthorchis viverrini* infection: an indication of the risk of opisthorchiasis transmission in non-endemic areas

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Abstract

Among the snail species acting as hosts for medically significant trematodes, only three taxa of *Bithynia* are responsible for transmitting the carcinogenic liver fluke *Opisthorchis viverrini* to humans in different geographical areas. Although *B. siamensis goniomphalos* is the primary species responsible for *O. viverrini* transmission in endemic areas, *B. siamensis siamensis* and *B. funiculata* remain potential hosts for transmission. This study objects to determine the susceptibility of *B. siamensis siamensis* and *B. funiculata* to *O. viverrini* to assess the risk of *O. viverrini* transmission in non-endemic areas. The snails of both species were first introduced to *O. viverrini* eggs, after which *O. viverrini* infection was investigated using specific PCR primers after a period of 1, 7, 14, 28, and 56 days post-infection (dpi). *Opisthorchis viverrini* infection in both *B. siamensis siamensis* and *B. funiculata* was high in the early period (1 and 7 dpi) while decreasing over time. It was also shown that the odds of susceptibility to *O. viverrini* infection in *B. siamensis siamensis* were 64.5% higher relative to the odds of susceptibility in *B. funiculata* ($P < 0.05$). Results of this study provide an early insight into the *Bithynia-Opisthorchis* relationship and thus have great potential to assess risk and raise awareness of opisthorchiasis in non-endemic regions, especially in regions endemic for *B. siamensis siamensis.*

Keywords *Bithynia* · *Opisthorchis viverrini* · Susceptibility · Cholangiocarcinoma

Introduction

Chronic opisthorchiasis caused by *Opisthorchis viverrini* (Leiper 1915) infection is generally accepted as the cause of bile duct cancer, cholangiocarcinoma (CCA) (IARC 2002; Bouvard et al. 2009). Prevalence of the *O. viverrini* infection has been reported in various parts of the Lower Mekong subregion countries, especially in Thailand and Lao PDR (WHO 1995; Sithithaworn et al. 2012; Aung et al. 2017). The northeast region of Thailand and the central and south regions of Lao PDR were found to have the highest prevalence than elsewhere in the countries (Jongsuksuntigul and Imsomboon 2003; Sithithaworn et al. 2012; Suwannatrai et al. 2018). In the transmission cycle, the parasite requires freshwater snails in Genus *Bithynia* (MolluscaBase 2021) and freshwater fish in Family Cyprinidae as first and second intermediate hosts, respectively. Humans get infected with the parasite by consuming raw or undercooked freshwater fish contaminated with infective metacercariae. Despite the freshwater

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fish's prominent role in transferring infective metacercariae to humans, *Bithynia* snails still play a crucial role in disease propagation as amplifier hosts, capable of releasing cercariae into the environment (Wykoff et al. 1965; WHO 1995; Sithithaworn and Haswell-Elkins 2003; Prasopdee et al. 2019). Three taxa of the *Bithynia* have been reported as natural hosts for *O. viverrini* and are in different geographical habitats. *Bithynia funiculata* (Walker 1927) is found in northern Thailand and Lao PDR, and *B. siamensis siamensis* (Lea 1856) in Central, South, and Northern Thailand; Southern Vietnam; Cambodia; and Myanmar. *Bithynia siamensis goniomphalos* (Morelet 1866) in Northeastern Thailand; Lao PDR; Southern Vietnam; and Cambodia (Brandt 1974; Harinasuta and Harinasuta 1984; Sithithaworn et al. 2008; Kulsantiwong et al. 2013; Miyamoto et al. 2014; Dao et al. 2017; Prasopdee et al. 2019). Among the habitats of the *Bithynia* species, the *B. siamensis goniomphalos* is in the regions where opisthorchiasis was highly reported. In a natural field survey, the prevalence of *O. viverrini* infection in *B. siamensis goniomphalos* was ranged from 0.45 to 8.37% (Sri-Aroon et al. 2005; Kiatsopit et al. 2012; Kulsantiwong et al. 2015; Prasopdee et al. 2020), whereas 0.3% and 1.6% were reported in *B. funiculata* and *B. siamensis siamensis*, respectively (Upatham and Sukhapanth 1980; Ngern-klun et al. 2006). Recently, in laboratory conditions, Prasopdee et al. (2015a) revealed susceptibility of *B. siamensis goniomphalos* to *O. viverrini* infection was 32.96%. In addition, it was shown that the infection rate decreased by the time post-infection increased. Although *B. siamensis goniomphalos* is the primary species responsible for *O. viverrini* transmission in endemic areas, *B. siamensis siamensis* and *B. funiculata* remain potential hosts for transmission. This study aims to determine the susceptibility of *B. siamensis siamensis* and *B. funiculata* to *O. viverrini* to assess the risk of *O. viverrini* transmission in non-endemic areas. This study would greatly benefit parasitologists, malacologists, and epidemiologists to comprehend the biology of snail-borne opisthorchiasis.

Materials and methods

Preparation of *Bithynia* snails

Bithynia siamensis siamensis and *B. funiculata* were collected from Klong Luang, Pathum Thani, Thailand (14° 37′ 32.7″ N, 100° 20′ 28.0284″ E) and San Kamphaeng, Chiangmai, Thailand (18° 30′ 39.744″ N, 99° 14′ 34.836″ E), respectively. The snails were sorted and identified based on shell morphology following available protocols (Brandt 1974; Upatham et al. 1983; Chitramvong 1992). Briefly, *B. siamensis siamensis* is a more slender shape, narrower umbilicus, and weaker carina when compared with *B. funiculata*. The shell of *B. funiculata* is a subovate conic (almost narrowly subovate conic), dull, green to reddish-brown (olive-brown). It is slightly larger than *B. siamensis siamensis*. The umbilicus is funnel-shaped with a strong carina (Fig. 1).

The snail samples were examined for trematode infection based on the cercarial shedding method (Prasopdee et al. 2020). Briefly, the snails were placed individually into plastic containers filled with 6 ml de-chlorinated tap water. Release of cercariae was induced by exposing the snails to 8-W electric light for at least 3 h, followed by covering the containers with black plastic sheets overnight. Uninfected snails of each species were used for further experiments.

Preparation of *O. viverrini* eggs

Two golden Syrian hamsters (*Mesocricetus auratus*) were experimentally infected with each 50 *O. viverrini* metacercariae. At 6 weeks post-infection, the infected hamsters were euthanized, and after that, *O. viverrini* adults were retrieved from biliary tracts and gallbladders. The recovered *O. viverrini* adults were washed several times with 0.85% sodium chloride solution and then subjected to obtain mature eggs from the distal part of the uterus under a stereoscope (Khampoosa et al. 2012). To undergo full maturation, the eggs were washed with distilled water and kept at room temperature

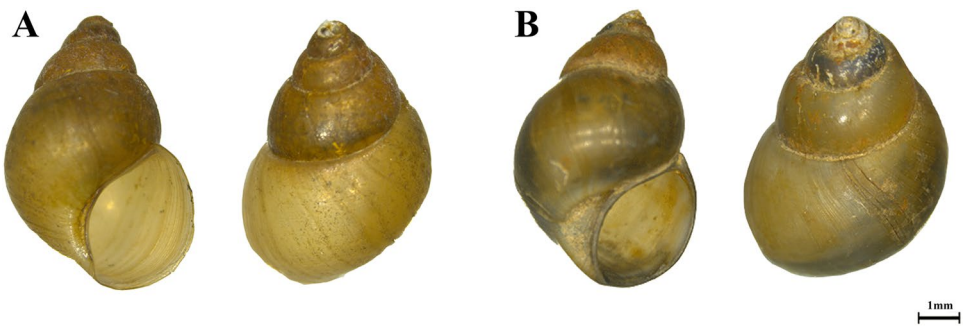

Fig. 1 Shell morphology of *Bithynia* species. **A** *B. siamensis siamensis*; **B** *B. funiculata*

for 2 weeks before experimental infection (Chanawong and Waikagul 1991).

Experimental infection and testing of susceptibility

For experimental infection, full-grown, medium-sized (0.61–1.00 cm) snails were used. In each species, 80 snails were placed individually in transparent plastic containers filled with 6 ml of de-chlorinated tap water and 50 embryonated *O. viverrini* eggs. The snails were infected in the plastic containers covered with porous lids and activated to ingest the parasite eggs by exposure to 8-W electric light for 24 h (Prasopdee et al. 2015a). Subsequently, the snails were washed and reared in the 15 × 20 cm glass containers according to their species, with a maximum of 50 snails per container density. Examination of egg hatching in snail feces was determined for snail infection (by observing the opening of the egg's operculum) (Khampoosa et al. 2012). The infected snails were raised at room temperature (approximately 28 °C) with a 12:12 dark and light cycle and fed synthetic snail food (Sumethanurungkul 1970). All exposed snails were checked weekly for shedding of cercariae for a total of 56 days. At 1, 7, 14, 28, and 56 days post-infection (dpi), 16 snails per time point were randomly selected and examined pre-patent infection using specific primer PCR if the snails were negative for cercarial shedding. The specific primer PCR was carried out as described by previous study (Prasopdee et al. 2015a). Briefly, soft body of the snail was retrieved and homogenized in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.2 mg/ml proteinase K) (Winnepenninckx et al. 1993). The snail homogenates were incubated at 55 °C for 6 h. Snail homogenate proteins were first precipitated with phenol/chloroform, centrifuged at 12,000 × g for 10 min at 4 °C followed by doubly precipitated with phenol/chloroform/isoamyl alcohol, centrifuged at 12,000 × g for 10 min at 4 °C. DNA was precipitated with isopropanol then washed twice with 70% ethanol followed by absolute ethanol. The DNA pellet was air-dried, re-dissolved with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), diluted to 10 ng/µl, and used as a template for PCR. The specific primers, OV-6F (5′-CTG AAT CTC TCG TTT GTT CA-3′) and OV-6R (5′-GTT CCA GGT GAG TCT CTC TA-3′) (Wongratanacheewin et al. 2001), were used to amplify the pOV-A6 specific region of 330 bp. The PCR reaction was carried out using a DNA Thermal cycler, performed in a final volume of 10 µl with 0.04 µl TaKaRa Ex Taq 250 U, 1 µl dNTP mixture, 1 µl 10 × Ex Taq buffer, 3 µl DNA sample, 5 pmol of each primer, and adjusted to 10 µl with distilled water. The PCR was carried out with cycling conditions of initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C denaturations for 1 min, 55 °C annealings for 1 min, and 72 °C extensions for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by 1.5% TBE agarose gel electrophoresis. Numbers of susceptible *Bithynia* snails to *O. viverrini* infection were obtained from the presence of either released cercariae or 330 bp specific band of PCR product as described in the previous study (Prasopdee et al. 2015a).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp, Armonk, NY, USA). The number of susceptible snails to *O. viverrini* infection was reported as % susceptibility. The association between dpi (predictor) and the susceptibility to *O. viverrini* infection (binary outcomes) of (1) *B. siamensis siamensis*, (2) *B. funiculata*, and (3) *Bithynia* spp. (*B. siamensis* and *B. funiculata*) were gauged by crude odds ratios obtained from binary logistic regression analysis. The binary logistic regression analysis was also used to find the association between the susceptibility to *O. viverrini* infection (binary outcomes) and species of the *Bithynia* snails (predictor).

Results

Determination of infectious status

There was no release of *O. viverrini* cercariae detected throughout the time-course of infection. However, the *Bithynia* snails were susceptible to the *O. viverrini* infection determined by specific primer PCR-based detection (Fig. 2).

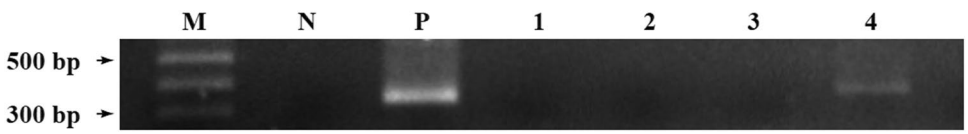

Fig. 2 Agarose gel electrophoresis showing 330 bp of PCR product generated with specific primers (pOV-A6) for *O. viverrini*. Lane M: 100 bp DNA ladder; N: *Bithynia* snail negative for trematode infection (based on cercarial shedding); lane P: *O. viverrini* adult; lane 1–4: experimental infection with *O. viverrini* in *Bithynia* snails

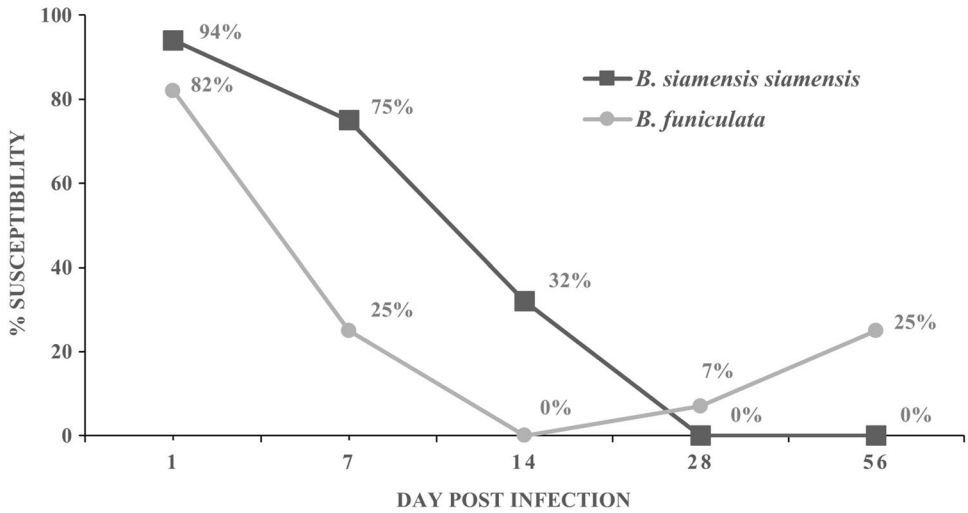

Fig. 3 Percent of snails of the *B. siamensis siamensis* and *B. funiculata* species infected by *O. viverrini* versus day post-infection (dpi)

Table 1 Association between infection and day post-infection of *B. siamensis siamensis*

Day post-infection	OR	95% CI
1 (reference)	$T_{wald} = 11.309$, $df = 4$, $P = 0.023$	
7	0.200	0.020–2.033
14	0.030*	0.003–0.297
28	n/a	n/a
56	n/a	n/a

Results of association are shown as odds ratio (OR)

n/a Not applicable (cannot be calculated because none of the snails were susceptible to infection)

*Significance at $P < 0.05$

Table 2 Association between infection and day post-infection of *B. funiculata*

Day post-infection	OR	95% CI
1 (reference)	$T_{wald} = 16.065$, $df = 4$, $P = 0.03$	
7	0.077*	0.014–0.417
14	n/a	n/a
28	0.015*	0.001–0.167
56	0.077*	0.014–0.417

Results of association are shown as odds ratio (OR)

n/a Not applicable (cannot be calculated because none of the snails were susceptible to infection)

*Significance at $P < 0.05$

Susceptibility of *Bithynia* snails to infection with *O. viverrini*

The highest percentage of susceptibility to *O. viverrini* infection of *B. siamensis siamensis* and *B. funiculata* was shown at 1 dpi with 94% and 82%, respectively. The susceptibility seemed to decrease as the dpi was increased (Fig. 3). The odds ratio (OR) for the association between susceptibility to *O. viverrini* infection of *Bithynia* snails and dpi is presented (Tables 1 and 2). In addition, there was evidence that *B. funiculata* was associated with a 64.5% decrease in odds of susceptibility to *O. viverrini* infection relative to *B. siamensis siamensis* ($T_{wald} = 4.737$, df = 1, $P = 0.03$).

B. siamensis siamensis vs *O. viverrini*

There was evidence of an association between dpi and susceptibility of *B. siamensis siamensis* to *O. viverrini* infection that at least two groups differed ($T_{wald} = 11.309$, df = 4, $P = 0.023$). Relative to day 1 (as baseline), there was a decrease in odds of susceptibility for 14 dpi ($P = 0.003$), showing an association with a 97% decrease in the odds of susceptibility. At 7 dpi, the susceptibility to infection was similar to 1 dpi ($P = 0.2$). Interestingly, there was no infection detected at 28 and 56 dpi.

B. funiculata vs *O. viverrini*

There was evidence of an association between dpi and susceptibility to infection of *B. funiculata* that at least two groups differed ($T_{wald} = 16.065$, $df = 4$, $P = 0.03$). Relative today 1 (as baseline), there was a decrease in odds of susceptibility for 7, 28, and 56 dpi ($P < 0.05$). There was evidence that 7 and 56 dpi were associated with a 92.3% decrease in the odds of susceptibility ($P = 0.003$). At 28 dpi, a 98.5% decrease in the odds of susceptibility was shown associated ($P = 0.001$). However, at 14 dpi, statistical evidence of susceptibility due to susceptible snails was not detected.

B. siamensis siamensis and *B. funiculata* (*Bithynia* spp.) vs *O. viverrini*

There was evidence of an association between dpi and susceptibility to infection of *Bithynia* spp. that at least two groups differed ($T_{wald} = 44.089$, $df = 4$, $P < 0.01$). Relative to day 1, there was a decrease in odds of susceptibility for every extra dpi (Table 3).

Discussion

Infectivity of *O. viverrini* in *Bithynia* snails was demonstrated to be temperature-dependent (Prasopdee et al. 2015a). In the present study, the chosen temperature of 28 ± 2 °C (room temperature) was applied to test the susceptibility to *O. viverrini* infection in *B. siamensis siamensis* and *B. funiculata* as it was demonstrated as an optimum range of temperature (22 to 34 °C) for *O. viverrini* infection in the sister taxa *B. siamensis goniomphalos* (Prasopdee et al. 2015a). In addition, as reported by the Thai Meteorological Department, this temperature is comparable with an average weather temperature of Thailand in the recent year 2019: 28 ± 1 °C. This was recorded as the highest average weather temperature of Thailand in the past 69 years (1951–2019), not only indicating global warming at effect, but also increasing the risk of snail-borne opisthorchiasis in humans and animals in the coming decades. Although the *Bithynia* snails have separate male and female individuals, the sex was not included in the current experimental infection setting since the susceptibility patterns to *O. viverrini* infection between male and female *B. siamensis goniomphalos* was not significantly different (Prasopdee et al. 2015a).

In a natural field survey for *O. viverrini* infection, among three taxa of *Bithynia* in Thailand, *B. siamensis goniomphalos* was reported to have the highest prevalence in comparison to other species (Kulsantiwong et al. 2015). Nevertheless, in experimental infection, *B. funiculata* and *B. siamensis siamensis* were more susceptible to *O. viverrini* than *B. siamensis goniomphalos* (Chanawong and Waikagul 1991). However, unlike the present study, the infection of *O. viverrini* in *Bithynia* snails of the previous studies was evaluated for only patent infection using a microscopic method cercarial shedding. In this study, observing of elicited *O. viverrini* cercariae and detecting specific bands of PCR product (330 bp) using specific primers to *O. viverrini*, both pre-patent and patent infections were determined throughout the time course of 1, 7, 14, 28, and 56 dpi. Interestingly, the susceptibility patterns of *B. siamensis siamensis* to *O. viverrini* infection demonstrated that the snail was highly susceptible to the parasite in the early period after exposure (94% and 75% for 1 and 7 dpi, respectively), before significantly becoming more resistant and finally, completely immune to the parasite during the later periods of infection. This trend is similar to the susceptibility patterns of *B. funiculata* to *O. viverrini* infection, where the susceptibility was found significantly higher at 1 dpi (82%) than other periods of infection (Fig. 1; Tables 1 and 2). This is under a previous study in *B. siamensis goniomphalos*, demonstrating that the odds of susceptibility were decreased when the dpi was longer (Prasopdee et al. 2015a). In this phenomenon, recent studies revealed several proteins and transcripts of *Bithynia* snails upon infection with *O. viverrini* involved in immune response (Prasopdee et al. 2014, 2015b; Suwannatrai et al. 2016). Moreover, as the snail hemocyte was reported involved in defense mechanism by killing invaded parasites (Adema et al. 1994; Humbert and Coustau 2001), several hemocyte proteins of *O. viverrini*-infected *Bithynia* snail were found differentially expressed at 1 and 2 dpi compared to uninfected *Bithynia* (Suwannatrai et al. 2016). This was possibly involved in the defense response of the *Bithynia* that could eliminate initial parasite invasion, which led to diminished and complete elimination of the parasite, respectively. Hence, the susceptibility to *O. viverrini* infection of the *Bithynia* was decreased over time. In addition, relative to day 1, the result presented herein demonstrated the odds of susceptibility of *Bithynia* spp. at 56 dpi was decreased approximately 98% (Table 3). This indicates less chance for further development and production of *O. viverrini* cercariae.

In natural field survey, the prevalence of *O. viverrini* infection (determined by detection of free cercariae) in *B. funiculata* was shown extremely low at 0.3% compared to 1.6% in *B. siamensis siamensis* (Upatham and Sukhapanth, 1980; Ngern-klun et al. 2006), likewise demonstrated in this study where the odds of susceptibility of *B. siamensis siamensis* were found 64.5% higher relative to *B. funiculata.* However, the *Bithynia* spp. act as the amplifier host for the transmission of *O. viverrini* to humans. In other words, the *O. viverrini* eggs are ingested by the snails, and miracidia hatch from the enclosed *O. viverrini* eggs in the snail digestive tract. The miracidia after that penetrate through the snail tissue and transform into sporocysts. The sporocysts undergo asexual reproduction through the redial stage to produce numerous free-swimming cercariae, which ultimately

Table 3 Association between infection and day post-infection of *Bithynia* spp.

Day post-infection	OR	95% CI
1 (reference)	$T_{wald} = 44.089$, $df = 4$, $P < 0.01$	
7	0.129*	0.036–0.471
14	0.022*	0.005–0.096
28	0.004*	0.000–0.037
56	0.017*	0.004–0.078

Results of the association are shown as odds ratio (OR)

*Significance at $P < 0.05$

infect and develop to infective metacercariae in intermediate fish hosts (Wykoff et al. 1965). Despite the low infection of *O. viverrini* in its *Bithynia* hosts, it is essential and sufficient for transmission. Thus, in addition to endemic areas, awareness of opisthorchiasis should be increased in other regions, especially in Central, South, and Northern Thailand; Southern Vietnam; Cambodia; and Myanmar, where the *B. siamensis siamensis* was found (Brandt 1974; Harinasuta and Harinasuta 1984; Sithithaworn et al. 2008; Kulsantiwong et al. 2013; Miyamoto et al. 2014).

Conclusion

We can conclude that susceptibility patterns of *B. siamensis siamensis* and *B. funiculata* to *O. viverrini* infection were similar where the susceptibility was high in early infection and decreased over time. Nevertheless, the *B. siamensis siamensis* was more susceptible to *O. viverrini* infection than *B. funiculata*. This provides a basis on the *O. viverrini*-intermediate snail host relationship that could help epidemiologists and parasitologists to indicate risk and raise awareness of opisthorchiasis in other regions besides the endemic areas.

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Author contribution Jutharat Kulsantiwong: methodology, investigation, data analysis, writing-original draft. Veerachai Thitapakorn: methodology, validation, writing-review and editing. Thanakrit Sathavornmanee: data analysis, writing-review and editing. Siraphatsorn Yusuk: investigation, visualization. Opal Pitaksakulrat: validation, writing-review and editing. Smarn Tesana: conceptualization, methodology, writing-review and editing. Sattrachai Prasopdee: conceptualization, methodology, investigation, data analysis, writing-original draft, funding acquisition.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval The Institutional Ethics Committee of Thammasat University approved all animal experiments in this study (clearance number 025/2561 and 026/2561).

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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