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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Parasitic infections in Malaysian aborigines with pulmonary tuberculosis: a comparative cross-sectional study

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Abstract

The geographical distribution of tuberculosis (TB) overlaps with various parasitic infections. Uncovering the characteristics of coinfecting parasites that potentially affect the host susceptibility to TB is pertinent as it may provide input to current TB therapeutic and prophylactic measures. The present study was aimed at examining the types of parasitic infections in TB patients and healthy TB contacts (HC) in Orang Asli, Malaysian aborigines, who dwelled in the co-endemic areas. Stool and serum samples were collected from Orang Asli who fulfilled the selection criteria and provided written informed consents. Selected parasitic infections in the two study groups were determined by stool examination and commercial serum antibody immunoassays. The prevalence of parasitic infections in TB and HC participants were 100% ($n = 82$) and 94.6% ($n = 55$) respectively. The parasitic infections comprised toxocariasis, trichuriasis, amoebiasis, toxoplasmosis, hookworm infection, ascariasis, strongyloidiasis, and brugian filariasis, in decreasing order of prevalence. Overall, helminth or protozoa infection did not show any significant association with the study groups. However, when the species of the parasite was considered, individuals exposed to trichuriasis and toxoplasmosis showed significant odds reduction (odds ratio (OR) 0.338; 95% confidence interval (CI) 0.166, 0.688) and odds increment (OR 2.193; 95% CI 1.051, 4.576) to have active pulmonary TB, respectively. In conclusion, trichuriasis and toxoplasmosis may have distinct negative and positive associations respectively with the increase of host susceptibility to TB.

Keywords Pulmonary tuberculosis · Parasitic infections · Healthy contact · Host susceptibility · Trichuriasis · Toxoplasmosis

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Introduction

Tuberculosis (TB), an airborne transmitted disease caused by *Mycobacterium tuberculosis* (*Mtb*), is one of the major four infectious diseases (apart from malaria, HIV/AIDS, hepatitis) that leads to millions of deaths each year (Chen et al. 2015; WHO 2018). Owing to the common predisposing risk factors such as low socioeconomic status and poor hygiene practice, the geographic distribution of TB overlaps with various parasitic diseases, which are either worldwide or region-specific (Torgerson et al. 2015; Tegegne et al. 2018). It is speculated that a parasitic coinfection could be a risk that increases the host susceptibility to TB, as the elicited immune response to counter a parasitic infection might in turn reduce the host TB immunity (Elias et al. 2008; Afifi et al. 2015; Mashaly et al. 2017; Taghipour et al. 2019).

The infection outcome upon exposure to *Mtb*, i.e. clearance, containment/latent infection and progressive TB, varies from one individual to another, wherein the host innate and

acquired immunity, mainly the Th1-driven-immune response, play a pivotal role in restricting the disease progression (O'garra et al. 2013). On the other hand, the existing data showed that infections with intracellular parasites such as *Toxoplasma gondii* stimulate a Th1-immune response, while infections with extracellular parasites, such as *Fasciola hepatica*, provoke a Th2-immune response (Miller et al. 2009). The effects of diverted Th1- and/or Th2-immune responses in the case of single or mixed parasitic infections on the host TB susceptibility remain unclear. Nevertheless, epidemiological findings on the coinfection characteristics from different geographical places prevalent for varied spectrums of parasitic infections could aid in filling the knowledge gap (Li and Zhou 2013).

In this context, one of the populations at risk of TB-parasite coinfection is the indigenous people (Narasimhan et al. 2013; Hotez 2014). The less than 150,000 Malaysian aborigines who are better known as Orang Asli are the minority of the Malaysian population who live in settlements mainly in the mountainous rainforest of the Main Range of Peninsular Malaysia. Most of them are poor and live isolated from the country's mainstream economy, in which 36.9% settle in the remote areas, 62.4% live in the rural areas and only 0.7% are found in urban areas (Noraini et al. 2018). The estimated TB incidence rate among the Orang Asli was 80.8 per 100,000[1] (KKLW 2014; Dony 2017) which was similar to the national incidence of 82.1 per 100,000 in the year 2014 (MOH 2015), while the mean prevalence of intestinal parasitic infections was about 64.1% (Lim et al. 2009). The present study was aimed at examining the distribution of selected parasitic infections or TB immunity of Orang Asli individuals who were diagnosed with pulmonary TB (PTB) and their healthy TB contacts (HC).

Material and method

Population and study settings

A cross-sectional study was conducted on Orang Asli communities to identify the types of parasitic infections prevalent among participants with PTB and their HC from September 2011 to May 2014. Based on sample size estimation formula, i.e. $N = Z_{\alpha/2}{}^2 * p*(1-p)/d^2$, the minimum sample size (N) for preliminary determination of parasitic infection profile among Orang Asli with TB and the healthy contacts were 42 subjects per group (inclusive of 20% dropout), where the estimated parasitic infection (p) was 90%, estimated margin of error (d) of 10% and alpha value (α) of 5% (Norhayati et al. 1997; Ahmed et al. 2011). Prior to recruitment, all participants with PTB and HC were interviewed to ensure that they did not have TB risk factors such as HIV, diabetes, malnutrition, tobacco smoking or alcohol consumption (WHO 2011). The absence of these TB comorbidities was established through interviews during visits at hospitals or Orang Asli settlements. Regarding the subject selection criteria, recruited PTB subjects were diagnosed according to the national TB diagnosis guideline, wherein all subjects were diagnosed clinically by TB signs and symptoms, chest X-ray, sputum AFB microscopy, and *Mycobacterium* culture and sensitivity assay. Only Orang Asli who had permanent residences in the conventional villages were recruited. All the PTB subjects who had recently finished (within 1 year) treatment were recruited at Orang Asli settlements, whereas inpatients who were still on direct observation short-course treatment programme (DOTS) were recruited at their respective hospitals. On the other hand, HC comprised individuals who were without antecedent PTB, stayed in the same house with a PTB patient, asymptomatic for PTB and negative for tuberculin skin test (TST < 10 mm). PTB and HC subjects with diseases associated with immunosuppression or under immunosuppressive treatment, pregnant women or those who were staying in the urban area were excluded from the present study. Nineteen sampling areas included aborigine settlements and the nearest hospitals, located in six different states of Peninsular Malaysia namely Kedah, Kelantan, Perak, Selangor, Johor and Pahang (Fig. 1). Participants were recruited with the help of hospital medical assistants, state TB liaison officers and officers of the Department of Orang Asli Development (JAKOA). The purpose and procedure of the study were explained to the participants prior to obtaining their written consents.

Stool sample collection and examination

The life cycles of *Toxocara canis*, *Entamoeba histolytica*, *T. gondii*, *Necator americanus*, *Ancylostoma duodenale*, *Ascaris lumbricoides*, *Strongyloides stercoralis* and *Brugia malayi* that were screened in this study involved lungs of the host except *Trichuris trichiura*. *T. trichiura* was included due to its consistent reported high presence at the Orang Asli settlements (Ahmed et al. 2011). Upon recruitment, a labelled wide-mouth screw-capped container was given to each participant who was instructed to return the container containing faecal samples on the following day. Considering the geographical constraint, the stool samples were kept in a cold icebox and transported to the Biomedicine Laboratory, School of Health Sciences, Universiti Sains Malaysia. A small portion of each collected stool sample was separately kept in − 20 °C for PCR analysis and the remainder was preserved in 10% formalin and kept in a cold room at 4 °C until microscopy examination by two experienced laboratory technicians.

[1] The numbers of TB cases and Orang Asli population in the year 2014 were obtained from the Tuberculosis and Leprosy Control Unit, Disease Control Division, Malaysia Ministry of Health and Malaysia Ministry of Rural and Regional Development, respectively.

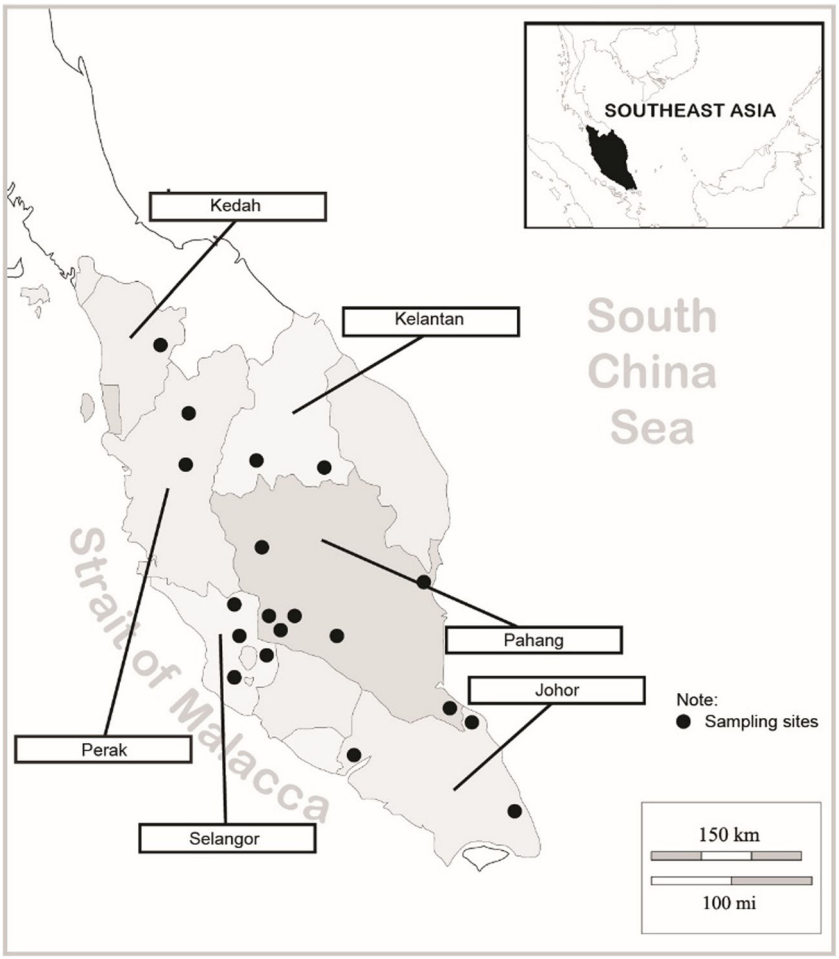

Fig. 1 Study sampling sites in 6 states of Peninsular Malaysia

Each sample was examined by direct wet mount (DWM), Kato-Katz (KK) and formalin-ether concentration (FEC) as previously described (Gotfred-Rasmussen et al. 2016; Wong et al. 2016). In brief, for DWM, a stool sample was spiked into separate droplets of normal saline and Lugol's iodine on a glass slide, mixed thoroughly with the aid of an applicator stick, covered with coverslips and examined under a microscope. For KK technique, the stool sample was first strained through a 0.1-mm screen and filled into the hole of a plastic template pre-fixed on a glass slide. The plastic template was then lifted and the sample on a glass slide was mixed with a drop of malachite green solution. The mixture was then covered with a cellophane membrane and observed under a microscope. For FEC, 1 g of stool sample was mixed with 5 mL of 10% formalin and strained through a 1-mm stainless steel sieve. The mixture was then transferred to a 15-mL centrifuge tube and centrifuged at 440×*g* for 2 min. The supernatant was discarded and the pellet was washed with 5 mL of 10% formalin for another two times. After washing, the pellet was resuspended in 5 mL of 10% formalin, added with 3 mL of ether solution and shook to mix vigorously. The mixture was centrifuged at 440×*g* for 2 min to form four layers namely, ether layer at the topmost, followed by the fatty plug, 10% formalin and sample pellet. The first three upper layers were removed with the aid of disposable Pasteur pipette. The pellet was resuspended in 500 μL of 10% formalin. About 100-μL sample was then examined under the microscope. Any stool sample yielding the evidence of any parasite ova, cysts and/or larvae was considered as positive. PCR was performed on all stool samples to detect *E. histolytica*, following the method described by Foo et al. (2012).

Serum sample collection and parasite serology

Blood specimens were collected in plain blood tubes by venipuncture, kept cooled in an icebox and transported to the processing laboratory for serum isolation. All serum samples were kept frozen at − 20 °C prior to the serological examination. Anti-parasite IgGs to *T. gondii*, *Strongyloides stercoralis*, *E. histolytica* and *T. canis* were detected using commercial kits namely Platelia™ TOXO IgG assay kit (Bio-Rad, France), SciMedx *Strongyloides stercoralis* serology microwell ELISA (IVD, USA), RIDASCREEN *Entamoeba histolytica* IgG (R-Biopharm,

Germany) and *Toxocara canis* IgG ELISA (IBL International, Germany), respectively. The assays were conducted according to the manufacturers' protocols. On the other hand, IgG4 specific to *B. malayi* was detected with an in-house IgG4-ELISA using recombinant *Bm*R1 antigen as described previously (Rahmah et al. 2001). In brief, a microtiter plate (96-wells, Maxisorp™ Nunc, Denmark) was coated with 100 μL of 20 μg/mL recombinant antigen in 0.02 M bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C, followed by 37 °C for 2 h and then washed 5 times with PBS + 0.05% Tween 20 (PBST). After blocking and washing steps, 100-μL serum sample/well (1:50 in PBS, pH 7.2) was incubated at 37 °C for 2 h, washed then followed by 30-min incubation with 100-μL monoclonal anti-human antibody IgG4-HRP (1:4500 in PBS; CLB, Netherlands). Following a final wash, the colour development was accomplished using ABTS substrate (Roche Diagnostics, Germany) for 30 min at 37 °C. The plates were read at a 410-nm test filter and 490-nm reference filter using an ELISA plate reader (DynaTech MR 5000, USA). All sera were tested in duplicates and results were expressed as optical density (OD) values. An OD of ≥ 0.3 was employed as the cutoff value for positivity; this was previously derived from the mean OD plus three standard deviations after testing 50-serum samples of normal individuals from a filariasis endemic area in Malaysia.

Data analysis

Demographic and laboratory findings were recorded and analysed using the SPSS programme. Categorical variables (i.e. gender, microscopic or serological findings) were reported as frequencies and percentages, while the continuous variable, age was reported as a mean and standard deviation. Logistic regression was employed to investigate the interactions between different parasitic diseases and PTB. Odds ratio (OR) and 95% confidence intervals (CI) were reported for logistic regression analysis.

Human ethical clearance

The study methodology was reviewed and approved by the Universiti Sains Malaysia Human Research Ethics Committee (JEPeM) [ref. no. USMKK/PPP/JEPeM[247.3(9)]], National Medical Research Register (NMRR) [ref. no. (2) dlm.KKM/NIHSEC/08/0804/P11-567] and Department of Orang Asli Development (JAKOA) [ref. no. JAKOA.PP.30.052 Jld.5(96)]. All positive stool and serological findings were sent to the local health authorities for their appropriate actions such as treating the infected individuals and advising them on the importance of proper hygiene practice.

Results

A total of 137 participants were recruited in the present study; there were 82 PTB patients and 55 HC. Their mean age was 36 ± 15 years old (Table 1). From the analysis, 97.8% of all participants were positive for any one of the parasitic infections and 89.6% of the positive cases were mixed infections, with up to 7 concomitant infections (Tables 2 and 3).

The presence of helminthic infections, as a whole, did not show difference between PTB and HC; however, when the species were considered, participants infected with *T. trichiura* showed significantly reduced odds to have active PTB (OR 0.338; 95% CI (0.166, 0.688); $p < 0.05$) (Table 2). None of the stool samples showed positive finding for *E. histolytica* and/or *Entamoeba* spp., either by coproscopy or PCR. *Giardia lamblia* cysts were present in some samples but prevalence of this protozoa was not included in this study. Meanwhile, amoebic serology showed 48.2% positive cases, suggesting exposure of the participants to invasive amoebiasis. Considering the infection with protozoa as a whole, there was no difference in terms of the prevalence between PTB and HC. However, when analysed based on species of the protozoa, participants with positive serological results for *T. gondii* infection showed increased odds to have active PTB (OR 2.193; 95% CI (1.051, 4.576); $p < 0.05$), as shown in Table 2. To further understand the interaction among the two infections and TB, a sub-analysis was performed as shown in Table 4. Participants with findings of *T. gondii* only (i.e. *T. trichiura* −ve and *T. gondii* +ve) showed significant four-fold increase odds of getting PTB. Cases with positive findings of *T. trichiura* only (i.e. *T. trichiura* +ve and *T. gondii* −ve) and the mixed (*T. trichiura* +ve and *T. gondii* +ve) showed about 40% lower odds of getting PTB but it was not significant with our small number of analysed samples.

Discussion

Although helminthic infections generally evoke the host Th2-immune response, the Th1-driven-immune responses against TB antigens in patients with *B. malayi* remained functional which suggest that the characteristics of the specific immune

Table 1 Demographic characteristics of study participants

	Total ($n = 137$)	PTB ($n = 82$)	HC ($n = 55$)
Age	36 ± 15	38 ± 16	33 ± 14
Gender			
a) Male	64 (46.7%)	42 (51.2%)	22 (40.0%)
b) Female	73 (53.3%)	40 (48.8%)	33 (60.0%)

Age was presented as mean age ± standard deviation. Gender was presented as frequency (percentage)

Table 2 Microscopic and serological findings

	Total	PTB	HC	OR (95% CI)	*p* value
Positive[a]	134/137 (97.8%)	82/82 (100.0%)	52/55 (94.5%)	4.673 (0.473, 46.138)	0.187
Mixed infection[b]	120/134 (89.6%)	72/82 (87.8%)	48/52 (92.3%)	0.600 (0.178, 2.024)	0.410
Helminthic infection	119/137 (86.9%)	70/82 (85.4%)	49/55 (87.5%)	0.714 (0.251, 2.033)	0.502
Protozoa infection	68/137 (49.6%)	39/82 (47.6%)	29/55 (51.8%)	0.813 (0.410, 1.612)	0.626
Helminthic infection					
(i) Microscopic findings					
1) *Trichuris trichiura*	68/137 (49.6%)	32/82 (39.0%)	36/55 (65.5%)	0.338 (0.166, 0.688)	0.003*
2) Hookworms	26/137 (19.0%)	16/82 (19.5%)	10/55 (18.2%)	1.091 (0.455, 2.620)	0.846
3) *Ascaris lumbricoides*	22/137 (16.1%)	12/82 (14.6%)	10/55 (18.2%)	0.771 (0.308, 1.934)	0.580
(ii) Serological Findings					
1) *Toxocara canis*	95/137 (69.3%)	58/82 (70.7%)	37/55 (67.3%)	1.176 (0.562, 2.457)	0.667
2) *Strongyloides stercoralis*	15/137 (10.9%)	9/82 (11.0%)	6/55 (10.9%)	0.990 (0.337, 3.008)	0.990
3) *Brugia malayi*	11/137 (8.0%)	9/82 (11.0%)	2/55 (3.6%)	3.367 (0.678, 15.743)	0.140
Protozoa infection					
(i) Microscopic findings					
1) *Entamoeba* spp.	Nil	Nil	Nil		
(ii) PCR findings					
1) *E. histolytica*	Nil	Nil	Nil		
(iii) Serological findings					
1) *Entamoeba histolytica*	66/137 (48.2%)	38/82 (46.3%)	28/55 (50.9%)	0.833 (0.420, 1.650)	0.600
2) *Toxoplasma gondii*	52/137 (38.0%)	37/82 (45.1%)	15/55 (27.3%)	2.193 (1.051, 4.576)	0.036*

[a] Any one of the parasites

[b] More than one infection

*Statistically significant, $p < 0.05$

response against different pathogens are differentiated (Sartono et al. 1996; Supali et al. 2010). In this regard, another study reported that tuberculin skin test (TST) responses are not influenced by helminthic infections (Zevallos et al. 2010). Chronic parasitic infections such as trichuriasis and giardiasis have been suggested to provide protection against *Mtb* infection (Watts et al. 2017). Conversely, the negative impact of helminth coinfection in TB patients has been reported, where the pulmonary radio-pathological findings were worse in the helminth coinfected TB patients (mainly with *S. stercoralis*), as compared with helminth-free patients (Resende Co et al. 2007). The existing evidence on TB-parasite coinfections has led to the hypothesis that the impact of parasite coinfection on the host susceptibility to TB is species-dependent (Watts et al. 2017). Numerous studies reported significant associations between parasitic infections (e.g. *T. gondii*, *T. trichiura*, *G. lamblia*, *A. lumbricoides*, *S. stercoralis* and hookworms) and TB (Tristao-Sa et al. 2002; Board and Suzuki 2015; Mashaly et al. 2017; Watts et al. 2017; Taghipour et al. 2019). Nonetheless, the incidence of multi-infection is not uncommon in TB-parasite co-endemic areas (Alemu and Mama 2017; Mashaly et al. 2017).

Table 3 Number of concomitant parasitological findings

No. of infections	Overall	PTB	HC
0	3/137 (2.2%)	0/82 (0.0%)	3/55 (5.5%)
1	14/137 (10.2%)	10/82 (12.2%)	4/55 (7.3%)
2	19/137 (15.3%)	12/82 (14.6%)	7/55 (12.7%)
3	36/137 (24.8%)	20/82 (24.4%)	16/55 (29.1%)
4	41/137 (29.9%)	26/82 (31.7%)	15/55 (27.3%)
5	15/137 (12.4%)	10/82 (12.2%)	5/55 (9.1%)
6	8/137 (4.4%)	4/82 (4.9%)	4/55 (7.3%)
7	1/137 (0.7%)	0/82 (0.0%)	1/55 (1.8%)

In the present study, the probability to have active TB was lower in individuals infected with *T. trichiura*, but not other helminthic infections. This finding is consistent with a previous report whereby the presence of *T. trichiura* infection was found to be associated with protection against *Mtb* infection based on TST results (Mashaly et al. 2017; Watts et al. 2017). According to the available data, it is hypothesized that individuals with *T. trichiura* infection might reduce the host susceptibility to TB. In a mouse model of infection with *Trichuris muris*, it was described that chronic infection with the parasite is associated with the presence of Th1-immune response and

Table 4 Association between *T. trichiura* and *T. gondii* findings

Types of infections	PTB	HC	OR (95% CI)	*p* value
T. trichiura −ve and *T. gondii* −ve	23 (28.0%)	15 (27.3%)	Ref	Ref
T. trichiura +ve and *T. gondii* −ve	22 (26.8%)	25 (45.5%)	0.574 (0.241, 1.366)	0.209
T. trichiura −ve and *T. gondii* +ve	27 (32.9%)	4 (7.3%)	4.402 (1.280, 15.140)	0.019*
T. trichiura +ve and *T. gondii* +ve	10 (12.2%)	11 (20.0%)	0.593 (0.202, 1.738)	0.341

*Statistically significant, $p < 0.05$

low level of Th2 polarization. In contrast, the presence of predominant Th2 response with low Th1 activation is associated with protection against *T. muris* (Bancroft et al. 1997; Grencis 2001). Taking into consideration these reports, it could be suggested that HC group in the present study has a predominant Th1-immune response, which protects against *Mtb* and predisposes to chronic *T. trichiura* infection, in contrast to the PTB patients which could have a predominant Th2-immune response, a factor associated with PTB in tropical areas and protection against the parasite (Rook 2010). Besides, interesting results from animal models demonstrated that the infection with *T. muris* establishes a cross talk between the gastrointestinal tract and lungs with the induction of a Th1 environment in the pulmonary tissue which suppress allergic reactions and potentially promotes protection against mycobacterial infections (Chenery et al. 2015). In future studies, this aspect should be explored with the determination of cytokine profiles of the groups under study.

The infection with *Helicobacter pylori* was reported to be associated with resistance to progression from latent to active TB, which suggests that concomitant infections could induce protective responses against TB (Perry et al. 2010). In the case of *T. trichiura*, the presence of shared epitopes, critical to protection, between the parasite and *Mtb* could be associated with protection against the infection. *T. trichiura* in endemic areas could be one of the components of the "self-antigenic universe" (classical microbiota and other organisms with relatively stable presence in the host), being part of the microbiota or the "familiar visitors" (organisms and substances which are often present in the host), producing protective cross-reactive responses against *Mtb* and/or adjuvant effect (Sarmiento et al. 2015).

Another major finding was that individuals infected with *T. gondii* showed increased odds to develop active TB. This finding is in agreement with a study by Mashaly et al. (2017) whereby Egyptian patients infected with *T. gondii* showed higher odds to have active TB. In addition, the study showed that the severity of TB (i.e. measured by the serum malondialdehyde concentration, an oxidative stress marker) was higher in patients coinfected with both microorganisms than those infected with *Mtb* alone. Association of *T. gondii* infection with TB in patients from China has also been reported by Zhao et al. (2017). Genetic polymorphism in the macrophage $P2X_7$ receptor has been reported to increase the susceptibility to TB and *T. gondii* infection in humans (Fernando et al. 2007; Niño-Moreno et al. 2007; Lees et al. 2010). According to Lees et al. (2010), the underlying mechanism seems to be associated with a deficit of macrophage activation and intracellular killing mediated by ATP. In the subset analysis of the interaction between positive findings of *T. trichiura* and *T. gondii*, subjects with positive findings of *T. gondii* alone showed higher odds of getting PTB. The odds of getting PTB was reduced in subjects with *T. trichiura* infection alone and the mixed infections, but bigger sample size would be needed to reach statistical significance. With enough study subjects, future studies could also focus on the determination of cytokine profiles in serum and stimulated peripheral blood mononuclear cell to investigate the possibility of a shift in Th1- and Th2-immune responses.

One aspect to be taken into consideration is the potential influence of anti-TB treatment on parasitic infections; in the case of DOTS, some studies had explored this aspect and did not find any influence of the TB treatment on the intestinal parasitic infections (Li et al. 2014; Li et al. 2015; Alemu and Mama 2017). There were some reports about the effect of some anti-TB drugs against different parasites (Conti and Parenti 1983; Pukrittayakamee et al. 1994; Araujo et al. 1996; Townson et al. 2000; Rao and Weil 2002; Townson et al. 2006; Mendez et al. 2009; Aditya et al. 2010; Debrah et al. 2011), but many of these studies were experimental and not implemented in the context of TB treatment. Although, rifapentine, a rifampicin derivative, had been reported with activity both "in vitro" and in a murine model of toxoplasmosis, in the current study, the use of rifampicin as part of DOTS did not show evidence of effect on toxoplasmosis; in fact, the PTB patients had a significant increase in this infection compared with healthy controls.

In conclusion, the results of this study indicated that coinfections of *T. trichiura* and *T. gondii* may have distinct positive and negative correlations respectively on the host susceptibility to PTB.

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