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PCR Detection of Entamoeba using Genus-Specific Primers from Orang Asli Schoolchildren in Perak

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Amoebiasis one of the intestinal parasitic infections (IPI) caused by protozoan parasite, *Entamoeba histolytica* which is still considered as the major cause of high morbidity and mortality, particularly in tropical countries. In Malaysia, the IPI is endemic among Orang Asli communities due to their low socioeconomic status and personal hygiene practices. However, there has not been a comprehensive prevalence study on Entamoeba distribution among children in rural communities throughout the nation. Thus, a cross-sectional study was carried out to detect the genus Entamoeba among schoolchildren in Perak. A total of 453 schoolchildren from selected Orang Asli primary schools have participated in this study. All samples were subjected to molecular detection and analysed using genus-specific PCR that amplifies the small subunit rRNA gene sequence of Entamoeba spp. The results showed 64.7% (293/453) samples were positive for Entamoeba. Of 19 statistically significant variables observed in the univariate analysis, two were retained as significant risk factors for Entamoeba spp. infection in multivariate analyses. These factors were: (i) presence of a toilet outside the house (AOR=1.58, CI=1.02, 2.43; *p*=0.041) and (ii) having gastroenteritis symptoms such as fever (AOR=0.41, CI=0.25, 0.68; *p*=0.001). In conclusion, the presence of Entamoeba species is still highly prevalent among children of Orang Asli communities in northwest of peninsular Malaysia. Hence, effective strategies such as providing health education specifically on personal hygiene practices and proper sanitation should be taught in schools and local communities to reduce the burden of this disease in this population.

Keywords: Entamoeba species, prevalence, risk factors, schoolchildren, Orang Asli

INTRODUCTION

The genus Entamoeba comprises seven different species namely, *Entamoeba histolytica, E. dispar, E. moshkovskii, E. bangladeshi, E. coli, E. harmanni* and *E. polecki*. All of these species can infect humans and remain confined to the intestinal lumen of asymptomatic carriers in most of the cases (Haghighi et al. 2018; Saidin et al. 2019). *E. histolytica, E. dispar* and *E. moshkovskii* are known as the Entamoeba complex, but only *E. histolytica* is well recognized as a pathogenic amoeba, associated with a wide range of clinical manifestations, from asymptomatic colonization to intestinal and extraintestinal amoebiasis (Bahrami et al. 2019). Previous studies have shown few factors such as socioeconomic, demographic, environmental and unhygienic practices facilitate the transmission of the disease (Quintanilla-Licea et al. 2014). The infection usually begins by ingestion of water or food that has been contaminated by *E. histolytica* cyst (Kantor et al. 2018). Currently, the number of reported cases of Entamoeba spp. infection due to the lack of safe drinking water, consumption of raw fruits and

vegetables has become a serious threat to public health in many developing countries especially among children (Bekele at al. 2017; Hind et al. 2020; Atabati et al. 2020; Alemu et al. 2020). In most cases, infected individuals are frequently present with diarrhea and majority of the cases resulting in malnutrition, abnormal physical growth and anemia (Tandukar et al. 2013; Gupta et al. 2020).

Amoebiasis is still considered the major cause of high morbidity and mortality, particularly in tropical and subtropical regions, where a large part of the impoverished communities does not have easy access to standard sanitary conditions (Carrero et al. 2020; Cui et al. 2019; Costa et al. 2018). Globally, 40 to 100 thousand deaths per annum were reported from 34 to 50 million symptomatic cases of amoebiasis (Shirley et al. 2018). The disease is endemic in certain areas of the world including West and South-East Africa, China, Mexico and Western portions of South America, and the Indian subcontinent (Prakash and Bhimji, 2017).

In Malaysia, amoebiasis showed a higher prevalence rate, ranged from 13.4-75% (Saidin et al. 2020; Anuar et al. 2012; Ngui et al. 2012; Anuar & Norhayati, 2011). However, the identification of Entamoeba infection particularly in children has not been fully investigated and organized control programs targeting transmission factors are lacking, thus warranted a study to provide some information about the epidemiology of the disease in the study area (Elyana et al. 2016; Ngui et al. 2012). Hence, this cross-sectional study was carried out to elucidate knowledge on the prevalence of Entamoeba and determine risk factors associated with the Entamoeba spp. infection among schoolchildren in Perak using PCR-based assay.

MATERIALS AND METHODS

Ethical consideration

The ethical approval was obtained from Sultan Idris Education University Human Research Ethics Committee (Ref No.: 2020-0153-01). The permission for fieldwork was also had obtained from the Ministry of Education Malaysia and the Department of Orang Asli Development (JAKOA) before the study commenced.

Sample and data collection

The stool samples were collected from 453 schoolchildren (March until November 2020) from five different Orang Asli primary schools in Perak namely Sekolah Kebangsaan Pos Bersih (SK Pos Bersih), Sekolah Kebangsaan Pos Tenau (SK Pos Tenau), Sekolah Kebangsaan Batu 14 (SK Batu 14), Sekolah Kebangsaan Pos Raya (SK Pos Raya) and Sekolah Kebangsaan Ulu Geruntum (SK Ulu Geruntum) using a wide-mouth screw-capped sterile container pre-labelled with code. All schoolchildren's ages ranged from 7 to 12 years. Socio-demographic background of the participants, environmental characteristics of living conditions, personal hygiene and gastrointestinal symptoms data were collected by interviewing all the subjects using a pre-designed questionnaire. Anthropometric measurements were assessed and body mass index (BMI) was calculated for all participants.

Genomic DNA extraction and PCR for identification of the genus-specific Entamoeba

Using AccuPrepTM Stool DNA Extraction Kit (Bioneer, South Korea), genomic DNA from 180-200 gm of stool samples were extracted following the manufacturer's instructions. The eluted DNA was stored at -20 ^{o}C, prior to PCR amplification. All extracted stool samples were subjected to molecular detection and analysed using genus-specific PCR that amplifies the small subunit rRNA gene sequence of Entamoeba spp. The Entam1, 5'-GTTGATCCTGCCAGTATTATATG-3' and Entam2, 5'-CACTATTGGAGCTGGAATTAC-3' were used to detect the Entamoeba genus with a product size of 550-bp. PCR reaction was performed in a final volume of 12.5 µl containing 6.25 µl NexPro e-PCR 2x Master Mix (Genes Laboratories, Korea), 1 µl of 10 pmole of each primer and 2 µl of extracted DNA samples. Nucleus-free water was added to a final volume of 12.5 µl. The PCR conditions were as follows: 94 ^{o}C for 3 min for initial denaturation, followed by 33 cycles of denaturing at 94 ^{o}C for 1 min, annealing at 56 ^{o}C for 1 min and extension at 72 ^{o}C for 1 min, followed by a final extension at 72 ^{o}C for 7 min. All PCR products were resolved on 1.5% agarose gel and visualized in a UV transilluminator (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The obtained data were analysed with the aid of SPSS version 23 (SPSS. Chicago, IL, USA). The general characteristics of the studied population, the prevalence of Entamoeba spp. infection and other categorical variables were expressed as frequencies and percentages. The Pearson's Chi-square (χ^2) test was used to

determine the association between Entamoeba infection and independent variables (demographic factor, socio-economic and environment factor). All independent variables that significantly associated with the prevalence of Entamoeba genus-specific in the univariate analysis with *p*-value <0.25 were further included in a multivariate logistical regression analysis to determine the strength of association between potential associated factors. Odds ratios (ORs), 95% confidence intervals (CIs) were calculated with the level of statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

General characteristics of population study

A total of 453 schoolchildren (49.7%, male and 50.3%, female) aged between 7 and 12 years participated in this study. Around 274 (60.5%) subjects who participated in this study were less than 10 years old and 179 (39.5%) were 10 years old or more (≥10). Based on the data, 436 (96.2%) of their father were worked as self-worker by collecting and selling forest products. Besides, some of the parents also working at rubber and oil palm plantation, on construction sites and unskilled labourers in factories while only 15 (3.3%) of the mother were recorded working. Therefore, without any stable income, a total of 279 (61.6%) families have earned less than RM500 per month and only 28 (6.2%) of the household have more than 8 members. More than half (52.1%, 236/453; mother and 50.8%, 230/453; father) of the parents have a low level of education i.e., less than 6 years of formal education. Unfortunately, all (100%) of the participants are still used untreated water originating from a nearby river for their domestic needs. 330/453 (72.8%) and 400/453 (88.3%) households present with toilet and electricity. Moreover, the majority 375/453 (82.8%) of participants kept dogs, cats and goats as their domestic animals. Some households also have very close contact with their animals and left them indoor. The details of the demographic and socioeconomic characteristics of the participants are presented in Table 1.

Prevalence of Entamoeba genus

Out of 453 stool samples, 293 were successfully amplified by PCR assay, resulted in an overall prevalence of 64.7%. The positive samples showed a band at 550-bp, which indicates Entamoeba spp. infection (Figure 1). The remaining 160 samples showed no presence of the expected PCR band in agarose gel. Therefore, these 35.3% samples are considered negative for the presence of Entamoeba spp. Figure 2 shows the prevalence of Entamoeba spp. according to schools, where SK Ulu Geruntum gives a higher prevalence rate (81.3%; 39/48) as compared to other schools. The lowest prevalence rate at 49.5% (51/103) was recorded at SK Pos Bersih. SK Pos Tenau, SK Batu 14 and SK Pos Raya represented the rate at 63.6% (14/22), 64.1% (84/131) and 70.5% (105/149), respectively.

Risk Factors associated with Entamoeba infection

Table 2 shows the univariate analysis for socio-demographic features of the participants and its association with Entamoeba genus infection. The finding shows gender and age groups (years) were not significantly associated with Entamoeba genus infection. Of the 293 participants who are positive with Entamoeba genus, 60.1% (176/293) were from schoolchildren less than 10 years old. Whereas, it was observed that 96.2% (282/293) of participants with non-working mothers were infected with the Entamoeba genus. The same number of infections (282/293) also recorded when the participants have a working father, but it was not significantly associated with the infection. Moreover, monthly household income was also recorded as not significant with these parasitic infections, (OR=0.98, CI=0.66, 1.46; p=0.926. Meanwhile, household member was resulted significantly associated with the detection, where families with more than eight members were 1.91 times (95% CI=0.89, 4.12; p=0.098) more likely to be detected positive with Entamoeba genus as compared to the families with less than 8 members. Then, participants with both parents having more than 6 years of formal education were also significantly associated with Entamoeba infection; OR=0.69, CI=0.49, 0.96; p=0.026 (mother's education level) and OR=0.67, CI=0.48, 0.93; p=0.017 (father's education levels), respectively. Similarly, the use of untreated water i.e., river, well and rain as the source of drinking water (OR=0.55, CI=0.0, 0.0; p=0.000) and the presence of toilet outside the house (OR=1.73, CI=1.13, 2.65; p=0.011) were also contributed as the risk factors of Entamoeba spp. infection. Concerning the presence of electricity, domestic animals and garbage disposal, it proved that all of these variables were not significantly associated with Entamoeba genus infection, as all of the *p*-

values more than 0.25. Therefore, based on this univariate analysis, families with more than eight members, parents' educational level, consuming untreated water (river, well & rain) and the presence of the toilet outside the house were independently associated with the infection.

Table 1: Demographic and socioeconomic characteristics of participants (n=453).

Characteristics	n (%)
Gender	
Male	225 (49.7)
Female	228 (50.3)
Age groups (years)	
7-9	274 (60.5)
10-12	179 (39.5)
Socioeconomic status	
Mother's/guardian occupation (Working mother)	15 (3.3)
Father's/guardian occupation (Working father)	436 (96.2)
Low monthly household income (<RM500)	279 (61.6)
Large family (>8 members)	28 (6.2)
Mother's education level (<6 years)	236 (52.1)
Father's education level (<6 years)	230 (50.8)
Source of drinking water (river, well & rain)	453 (100)
Presence of toilet in the house	330 (72.8)
Presence of electricity	400 (88.3)
Presence of domestic animals	375 (82.8)

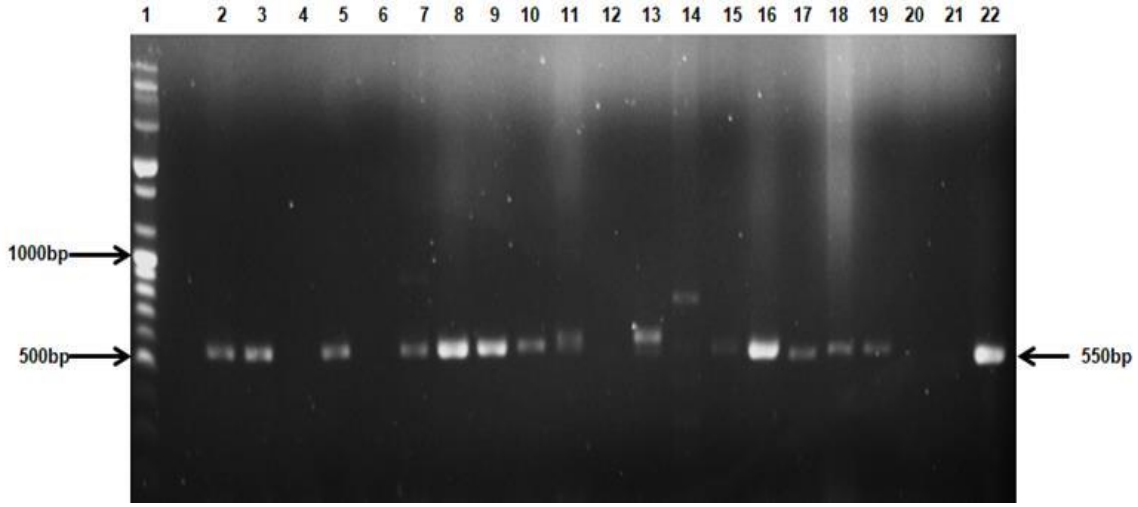

Figure 1: The representative image shows the agarose gel electrophoresis of the amplification of the Entamoeba DNA. The 550-bp band depicts specific amplification of the Entamoeba genus. Lane 1 is the 100-bp ladder molecular weight marker. Lanes 2, 3, 5, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18 and 19-positive test samples, Lanes 4, 6, 12 and 20-negative test samples, Lane 21: negative control; Lane 22: positive control (*E. histolytica*- HM1:IMSS).

Table 2: Socio-demographic features of the participants and its association with Entamoeba genus infection (n=453).

Variables	Entamoeba genus			
	No. of examined	n (% positive)	OR (95%, CI)	*p*-value
Gender				
Male	225	151 (51.5)	1	0.282
Female	228	142 (8.5)	1.24 (0.84, 1.82)	
Age group (years)				
< 10	274	176 (60.1)	1	0.806
≥ 10	179	117 (39.9)	0.95 (0.64, 1.41)	
Mother's/guardian occupation				
Working	15	11 (3.8)	1	0.479
Non-working	438	282 (96.2)	1.52 (0.48, 4.86)	
Father's/guardian occupation				
Working	436	282 (96.2)	1	0.998
Non-working	17	11 (3.8)	1.00 (0.36, 2.75)	
Monthly household income				
<RM500	278	180 (61.4)	1	0.926
>RM500	175	113 (38.6)	0.98 (0.66, 1.46)	
Household members				
<8	425	274 (95.2)	1	0.098
≥ 8	28	14 (4.8)	1.91 (0.89, 4.12)	
Mother's education level				
Non-educated	21	12 (4.1)	1	0.026
Primary school	215	129 (44.0)	0.69 (0.49, 0.96)	
Primary & secondary schools	217	152 (51.9)		
Father's education level				
Non-educated	19	12 (4.1)	1	0.017
Primary school	211	123 (42.0)	0.67 (0.48, 0.93)	
Primary & secondary schools	223	158 (53.9)		
Source of drinking water				
Treated and piped water	0	0	1	0.000
River, well & rain	453	293 (64.7)	0.55	
Presence of toilet				
In house	330	225 (76.8)	1	0.011
Outside house	123	68 (23.2)	1.73 (1.13, 2.65)	
Presence of electricity				
Yes	400	259 (88.4)	1	0.932
No	53	34 (11.6)	1.03 (0.57, 1.87)	
Presence of domestic animals				
Yes	375	243 (82.9)	1	0.907
No	78	50 (17.1)	1.03 (0.62, 1.72)	
Garbage disposal				
Burning	449	289 (98.6)	1	0.999
Buried on soil	0	0	0	
Outdoor	4	4 (1.4)		

CI=Confidence interval; OR, Odds ratio, reference group marked as OR=1.

Table 3: Univariate analysis of selected environmental factors and subjects clinical symptoms associated with Entamoeba genus infection among schoolchildren (n=453).

Variables	Entamoeba genus			
	No. of examined	n (% positive)	OR (95%, CI)	*p*-value
Washing hands before eating				
Yes	445	287 (98.0)	1	0.542
No	8	6 (2.0)	0.61 (0.12, 3.04)	
Washing hands after playing with soil				
Yes	443	288 (98.3)	1	0.333
No	10	5 (1.7)	1. 86 (0.53, 6.52)	
Indiscriminate defecation				
River/bush/open space	58	37 (12.6)	1	0.880
Toilet	395	256 (87.4)	0.96 (0.54, 1.70)	
Washing hands after defecate				
Yes	443	289 (98.6)	1	0.113
No	10	4 (1.4)	2. 82 (0.78, 10.13)	
Washing hands after using toilet				
Yes	436	282 (96.2)	1	0.998
No	17	11 (3.8)	1.00 (0.36, 2.75)	
Washing vegetables or fruits before eating				
Yes	426	280 (95.6)	1	0.069
No	27	13 (4.4)	2.07 (0.95, 4.51)	
Consuming raw vegetables				
Yes	237	146 (49.8)	1	0.152
No	216	147 (50.2)	0.75 (0.51, 1.11)	
Boiled water before drinking				
Yes	404	266 (90.8)	1	0.198
No	49	27 (9.2)	1.49 (0.81, 2.73)	
Eating fresh fruits				
Yes	413	263 (89.8)	1	0.157
No	40	30 (10.2)	0.58 (0.28 ,1.23)	
Cutting nails periodically				
Yes	368	240 (81.9)	1	0.619
No	85	53 (18.1)	1.13 (0.70, 1.85)	
Like to suck fingers				
Yes	72	45 (15.4)	1	0.673
No	381	248 (84.6)	0.89 (0.53, 1.51)	
Wearing shoes when outside				
Yes	396	258 (88.1)	1	0.580
No	57	35 (11.9)	1.18 (0.66, 2.08)	
Wearing shoes when inside				
Yes	97	59 (20.1)	1	0.371
No	356	234 (79.9)	0.81 (0.51, 1.29)	
Washing hands after playing with domestic animals				
Yes	396	251 (85.7)	1	0.131
No	57	42 (14.3)	0.62 (0.33, 1.15)	
Bathing in river or lake				
Yes	296	185 (63.1)	0.76 (0.50, 1.14)	0.183
No	157	108 (36.9)	1	
Diarrhoea				

Yes	113	67 (22.9)	0.74 (0.47, 1.14)	0.167
No	340	226 (77.1)	1	
Vomiting				
Yes	90	44 (15.0)	0.44 (0.27, 0.70)	0.001
No	363	249 (85.0)	1	
Nausea				
Yes	79	37 (12.6)	0.41 (0.25, 0.67)	0.000
No	374	256 (87.4)	1	
Stomach ache (abdomen)				
Yes	128	72 (24.6)	0.61 (0.40, 0.92)	0.019
No	325	221 (75.4)	1	
Watery stool				
Yes	78	42 (14.3)	0.58 (0.35, 0.96)	0.029
No	378	251 (85.7)	1	
Bloody or slimy stool				
Yes	18	7 (2.4)	0.33 (0.13, 0.87)	0.025
No	435	286 (97.6)	1	
Fever				
Yes	75	34 (11.6)	1.38 (0.23, 0.63)	0.000
No	378	259 (88.4)	1	

CI=Confidence interval; OR, Odds ratio, reference group marked as OR=1.

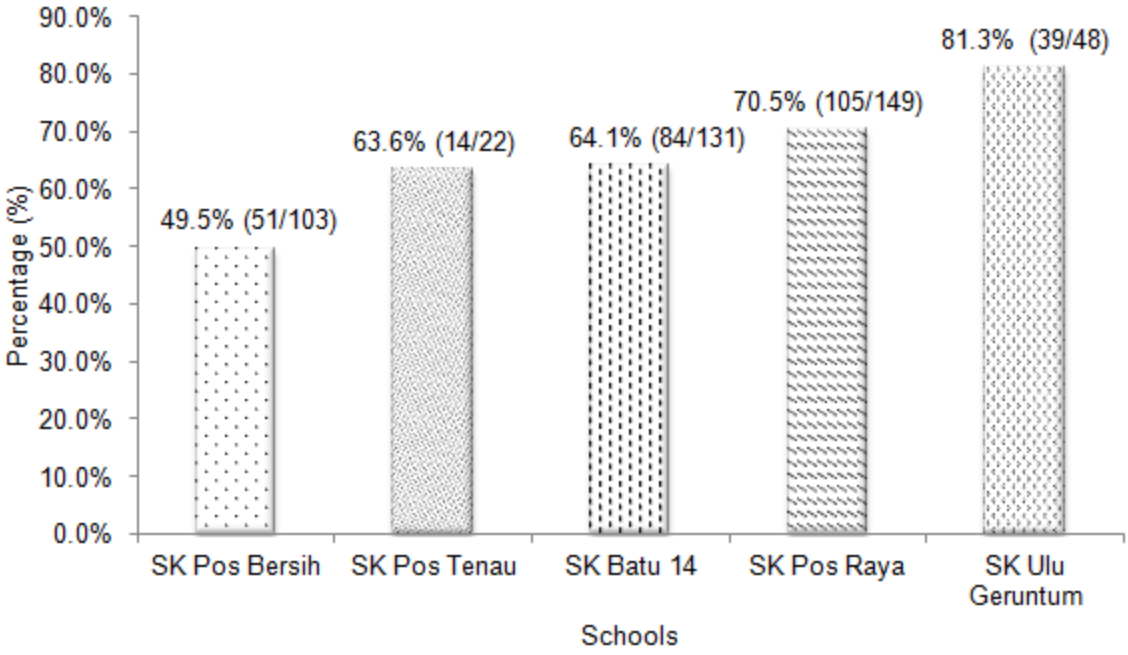

Figure 2: Prevalence of Entamoeba according to schools detected using genus-specific PCR method.

The data of univariate analysis of environmental factors and subject clinical symptoms associated with Entamoeba genus infection are shown in Table 3. The results show that not washing hands after defecate (OR=2.82, CI=0.78, 10.13; *p*=0.113), not washing vegetables or fruits before eating (OR=2.07, CI=0.95, 4.51; *p*=0.069), not consuming raw vegetables (OR=0.75, CI=0.51, 1.11; *p*=0.152), not boiled water before drinking (OR=1.49, CI=0.81, 2.73; *p*=0.198), not eating fresh fruits (OR=0.58, CI=0.28, 1.23; *p*=0.157), not washing hands after playing with domestic animals (OR=0.62, CI=0.33,1.15; *p*=0.131) and bathing in river or lake (OR=0.74, CI=0.47, 1.14; *p*=0.183) were significantly associated with Entamoeba spp. infection.

Besides, Entamoeba spp. infection were also detected to be significantly associated with all of the clinical symptoms which include participants had diarrhoea (OR=0.74, CI=0.47, 1.14; *p*=0.131), and gastroenteritis symptoms such as vomiting (OR=0.44, CI=0.27, 0.70; *p*=0.001), nausea (OR=0.41, CI=0.25, 0.67; *p*=0.000), stomach ache (OR=0.61, CI=0.40, 0.92; *p*=0.019), watery stool (OR=0.58, CI=0.35, 0.96; *p*=0.029), bloody/slimy stool (OR=0.33, CI=0.13, 0.87; *p*=0.025) and fever (OR=1.38, CI=0.23, 0.63; *p*=0.000).

In the multivariate logistic regression analysis, two variables were identified as a significant risk factor of Entamoeba infection among Orang Asli schoolchildren (Table 4). The presence of a toilet outside the house was 1.58 more likely to be infected with Entamoeba spp. (AOR=1.58, CI=1.02, 2.43; *p*=0.041) as compared to the presence of a toilet inside the house. On the contrary, participants who had gastroenteritis symptoms such as fever reduce the risk to be infected (AOR=0.41, CI=0.25, 0.68; *p*=0.001) with Entamoeba spp. as compared to other clinical symptoms.

Table 4: Multivariate analysis of risk factors associated with Entamoeba genus infection among schoolchildren (n=453)

Factors	Adjusted OR (95% CI)	*p*-value
Overall		
Socio-demographic factor		
Household member (≥ 8 members)	2.13 (0.92, 4.98)	0.080
Mother' education level	0.68 (0.16, 2.96)	0.611
Father's education level	1.15 (0.24, 5.43)	0.859
Presence of toilet outside house	1.58 (1.02, 2.43)	0.041*
Source of drinking water (river, well & rain)	-	-
Personal hygiene factors		
Not washing hands after defecate	3.86 (0.99, 15.09)	0.053
Not washing vegetables or fruit before eating	1.88 (0.79, 4.47)	0.152
Not consuming raw vegetables	0.78 (0.51, 1.21)	0.226
Not boiled water before drink	1.30 (0.66, 2.56)	0.451
Not eating fresh fruit	0.47 (0.21, 1.06)	0.070
Not washing hands after playing with domestic animals	0.65 (0.33, 1.28)	0.212
Bathing in river or lake	1.06 (0.67, 1.66)	0.812
Clinical symptoms		
Diarrhoea	1.58 (0.84, 2.98)	0.115
Vomiting	0.88 (0.32, 2.43)	0.808
Nausea	0.53 (0.19, 1.48)	0.223
Stomach ache (abdomen)	1.05 (0.55, 2.00)	0.891
Watery stool	1.30 (0.61, 2.77)	0.497
Bloody or slimy stool	0.63 (0.20, 1.94)	0.418
Fever	0.41 (0.25, 0.68)	0.001*

CI, confidence intervals. OR, Odds ratio.
*Significant association (*p* <0.05).

DISCUSSION

This study revealed a high prevalence of 64.7% (293/453) among Orang Asli schoolchildren in Perak, Malaysia using the Entamoeba genus-specific molecular technique. The prevalence of Entamoeba spp. reported by this current study was comparable with other reported studies in Malaysia. For instance, a study on the prevalence and distribution of anti-amoebic IgG antibodies among Orang Asli in Peninsular Malaysia (Kedah, Kelantan, Perak, Pahang, Selangor and Johor) detected 71% (266/375) were seropositive for amoebiasis by anti-amoebic IgG antibody assay. In their study, Orang Asli from rural areas was recorded with higher seropositivity 76% (192/254; p=0.002) as compared to that of 65% (69/106) from near urban areas and urban areas [36% (4/11)] (Wong et al. 2016). These observations might be contributed to the fact that the Orang Asli community from rural areas still had low socioeconomic, education and health services which had exposed them to getting infected by this disease. A much higher prevalence rate of 86.2% was reported using real-time PCR assay among 334 participants from seven different Orang Asli settlements in Pulau Carey, Hulu Yam, Hulu Langat, Bentong and Kuala Kangsar. However, slightly lower positive cases (80%) were observed when tested with nested PCR assay (Lau et al. 2013). This finding is expected since the real-time PCR assay was more sensitive and effective as compared to the nested PCR molecular method.

With regards to the socio-economic factors, our study found out gender and age groups were not significantly associated with Entamoeba infection. This is in line with the previous studies which recorded no significant association between gender or age groups with Entamoeba-complex species (Anuar et al. 2012). Besides, a study in a rural community of Venezuela recorded no prevalence of *E. histolytica/E. dispar* with the gender of the participants (Incani et al. 2017). The result of this study, in contrast to previous studies, showed gender and age groups were found related to the prevalence of *E. histolytica/E. dispar* among Iraqi provinces. The study determined males with the age group of 15-40 were found with a higher prevalence of getting Entamoeba infection due to few factors such as exposure to contaminated food, water and soil at their worksites, involvement in various outdoor activities (Al Saqur et al. 2017; Sahiminet al. 2019). Similarly, no significant difference was also noted between Entamoeba infection with families consist of more than 8 members, parents' education levels and source of drinking water. These findings are in contrast to the data reported by Chin et al. (2016) who found that having a large family significantly became the potential risk for transmission of intestinal parasitic infection among Orang Asli communities in Selangor, Malaysia. Similarly, a study in Sebha, Libya by ESalem et al. (2017), confirmed the infection of pathogenic *E. histolytica* was slightly higher in the large family compared to the small family.

These results indicate the overcrowded household and have relatively close contact with family members would increase the probability of getting infected by this parasite. However, in our study, we observed that most of the participants with large families' member were not significantly infected with Entamoeba. The possible explanation for this finding might be due to the fact that these families tend to live in two different houses (traditional house and modern house) and thus, this habit could reduce the risk of getting infected with Entamoeba. Although most of the households kept domestic animals like dogs and cats, our results show participants in this study were less to be infected with Entamoeba spp. This result corroborates with the finding from a study in rural Terengganu, Malaysia as it reported the presence of domestic animals among Orang Asli communities was one of the potential risk factors for getting infected with intestinal parasitic. They also examined 77.1% (OR=3.13, CI=1.57, 6.23; p=0.001) of the participants were having domestic animals such as dogs and cats in their household (Elyana et al. 2016). It is possible for the participants to use their unwashed hands and getting infected by Entamoeba spp. after touching their domesticated animals. A study conducted by Ngui et al. (2020) and Schär et al. (2014), reported the domestic animals could act as parasite carriers and reservoir hosts for potential zoonotic disease.

Interestingly, our study also identified most of the potential risk factors of getting Entamoeba spp. infection is associated with poor hygiene practices among schoolchildren. This finding is in agreement with other studies in Malaysia and other countries which showed that the prevalence rate of Entamoeba infection was particularly contributed by the environment and unhygienic practices. As an example, a cross-sectional study in Yemen found out not washing vegetables before consuming was 2.05 times higher (CI=1.32, 3.19; p=<0.001) in getting *E. dispar* infection (Al-Areeqi et al. 2017). A similar study

was previously reported, 74.3% (220/296) Orang Asli schoolchildren in Lipis, Pahang Malaysia who not washing their hands after defecations, was at higher risk of getting Entamoeba infection. Significantly, it indicates that participants who not washing hands after defecation was 1.64 times more likely to be infected with Entamoeba spp. as compared to participants who washing hands after defecation (CI=1.08, 2.37; *p*=0.017). Whereas, 80.5% (211/262) of the participants also were infected with Entamoeba spp. after consuming unwashed vegetables (Al-delaimy et al. 2014). In a recent cross-sectional study, eating raw and unwashed vegetables were also recognized as the risk factors that elevated the prevalence of intestinal parasitic infection among schoolchildren of Jawi Primary School, Ethiopia. The study examined 5.9% (24/406) participants in age between 6 to 18 years positive with *E. histolytica/E. dispar* infections (Sitotaw et al. 2017). This was due to the ingestion of cysts from the contaminated vegetables from the ground or dirty hands. Moreover, the cyst of Entamoeba can survive outside the host (soil/water/food) especially under moist conditions on the latter for a long period. Besides, vegetables such as spinach and cabbage were proved to be more prone to get contamination as compared to fruits. This was also contributed to the fact that vegetables have more uneven and rough surfaces, as well as their edible parts grow much closer to the soil as compared to fruits (Alemu et al. 2020). Majority of the infected participants in this current study used non-boiled water for drinking, not eating fresh fruits, not washing hands after playing with domestic animals and bathing in a river or lake. A similar association was also reported in Cambodia, where 50.3% (155/308) of schoolchildren having non-boiled water and get infected by intestinal parasites. This was attributed to the fact that most of the families do not have treated tap water or a water purification system in their household (Liao et al. 2017). During our school visits, we also observed most of the schoolchildren had good hygiene practices such as always washing hands, consuming boiled water for drink and consume fresh fruits. However, some teachers and parents witnessed most of the participants directly use untreated tape water (mostly from the nearby river) as their drinking water whenever they at their house or outside the classroom at the schools. The use of untreated water from rivers or lake was one of the reasons why this population having infected with Entamoeba spp. Therefore, these problems retain in contributing to the high infection rate among Orang Asli schoolchildren in this current study. However, we confirmed that this variable was not retained in the multivariate analysis and significantly not contributed to the risk of getting infected with Entamoeba spp. In multivariate analysis, we found the presence of a toilet outside the house is 1.58 times higher in getting the infection while having a fever is less risk to be infected by Entamoeba. As proved by questionnaire analysis and observation, we detected 27.2% (123/453) of the participants did not have proper sanitary facilities in their household. This could be the main reason they defecate around the house and it is such a common practice in the communities. Our findings are in agreement with another report, which found the absence of the toilet in the house demonstrated a 2.47 greater risk of contracting Entamoeba infection (Al-delaimy et al. 2014). Similar finding was also reported among villagers in Kalena Rongo, South West Sumba, Indonesia where there was an association between the absence of a toilet in the house and Entamoeba spp. infection (Sungkar et al. 2015). We also found clinical symptoms such as fever are less likely to associate with the Entamoeba infection and. Similar observation have been reported in a retrospective study carried out among 6,867 fever diagnosed returning travelers in two “post travel” clinic in Israel. Only 0.77% were detected positive for amoebiasis and 26% of them were diagnosed with ALA (Lachish et al. 2016). A retrospective observational study in Germans Trias i Pujol University Hospital, Badalona and Vall d'Hebron University Hospital at Barcelona city were also recorded 24% (12/50) of amoebic colitis patients having fever symptom (Roure et al. 2019). A study carried out by Naous et al. (2013) among pediatric population revealed that 94.2% of children infected with *E. histolytica* had fever. However, there are still no studies that identify the association between infection of Entamoeba spp. and fever, thus warrant further investigation.

There are some limitations in the current study. First and foremost, we only managed to collect a single stool sample from each subject that participated in this study. We believed some of the indigenous communities especially parents refused to give their children stool samples more than one time due to their cultural beliefs, even after general explanations on the purpose of this study. Therefore, it is reasonable to expect that the actual prevalence is underestimated due to the intermittent nature of mature cyst excretion in

the stool. Moreover, this study was performed based on the detection of genus-specific PCR and hence, future studies need to be conducted using species-specific primers in order to identify the true prevalence of Entamoeba spp. infection.

CONCLUSION

In conclusion, this study observed the presence of Entamoeba species is still high among children, especially in rural populations. Moreover, the results confirmed that the presence of a toilet outside the house and fever are related to Entamoeba infection. Therefore, to determine the accuracy of the species infection, positive samples from Entamoeba genus-specific PCR should be further investigated using single-round PCR using species-specific primers develop for differential diagnosis of *E. histolytica, E. dispar* and *E. moshkovskii.* Thus, this observation will then contribute to control the growing infection among schoolchildren and become the main reason for planning and formulating effective control measures and strategies by authorities to cater to the need of this community in Malaysia.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NIT, SS and AAB conceived and designed the experiments, data analysis and wrote the manuscript. NHO and RN revised and reviewed the manuscript. All authors read and approved the final version.

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