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Morphological and Morphometric Analysis of *Trypanosoma lewisi* and *Toxoplasma gondii* in Malang City, Indonesia Rats

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Malang

Rat

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ABSTRACT

Rats are reported to be the intermediate hosts and reservoirs of several zoonotic protozoal diseases. *Trypanosoma lewisi* is commonly reported in rats' blood and is considered non-pathogenic protozoa in humans. However, some countries documented several cases in humans with *T. lewisi* infection. Another zoonotic protozoon that develops in rats and can be transmissible to humans is *Toxoplasma gondii*. We intended to present the morphology and morphometry of *T. lewisi* and *T.gondii* in wild rats collected around Malang City to explore the potential risk of transmission nearby. The rats were collected using single live traps followed by identification, sexing, age approximation, and body morphometry. All specimens were euthanized according to the standard procedure followed by blood and peritoneal fluid collection. The fluid smear preparation and Giemsa staining were performed to detect the presence of *T. lewisi* and *T. gondii*. Morphologic and morphometric analyses were conducted using ImageJ software. Among the collected 50 collected rats, 23 were identified as *Rattus norvegicus* (46%), 22 as *Rattus rattus* (44 %), and 5 as *Mus musculus* (1%). In the case of protozoans infection, ten individuals were infected with *T. Lewisi* (20%) from the blood smear check, whereas peritoneal fluid smear examination revealed an infection of *T. gondii* in a specimen (2%). Results of the study proved

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trypanosomiasis and toxoplasmosis in wild rats in Malang City. Though the clinical significance to human and public health impact is questionable, further research and surveillance of rodent-borne parasitic pathogens will provide more information for pre-emptive action.

1 Introduction

Rodents are small mammals, with predominant habitats ranging from natural to urban areas. Rodent species, such as *Rattus sp.* (black rat) and *Mus musculus* (house mouse), are commonly found in Indonesia. Rats are found near human habitation, and they play a crucial role as a reservoir or definitive host of many zoonotic diseases (Gratz 1994). Urban rats potentially bring pathogenic parasites to humans and spread through uptrend contact with a human because of urbanization (Sumangali et al. 2007). The rodent population in an urban area may increase the exposure and susceptibility to rat-borne diseases (Neves Souza et al. 2021). Recently, an increased incidence of cases closely related to zoonotic parasites has been recorded in several countries (WHO 2019).

Trypanosoma lewisi is a hemo-protozoan unicellular parasite and is known as non-pathogenic in rats. According to Sarataphan et al. (2007), *T. lewisi* has zoonotic potential, evidently reported in Thailand. In 2005, an infant was infected by *T. lewisi* and presented general symptoms of fever, cough, and anorexia. Another protozoal disease is Toxoplasmosis, caused by *Toxoplasma gondii*, a tissue, and intracellular parasite. This parasite is categorized as a heterogenous parasite, infecting warm-blooded animals, including domestic and wild animals, livestock, and humans (Weiss and Kim 2007). Further, *T.gondii* has veterinary and medical importance due to congenital diseases in animals and humans. *T. lewisi* and *T. gondii* infecting rats have different morphometry depending on their host and geographic distribution. This study intended to determine the morphologic and morphometric analysis, geographical distribution, and disease proportion of *T. lewisi* and *T. gondii* in wild rats in Malang. Malang has a high population and distribution of syntrophic animals, indicating a high potential for zoonotic diseases transmitted by rats. People living close to rodents are potentially exposed to zoonotic protozoa infection. This study also aimed to explore the possibility of zoonotic protozoan risk associated with rats in the study field area.

2 Materials and methods

2.1 Animals Ethic

Animal Care Committee Universitas Brawijaya adequately approved the protocol of animal ethics. The approval numbers are 090-KEP-UB-2021.

2.2 Research and study area

The location of the study area is in Malang, East Java, Indonesia. Rats were caught from human residences in urban areas and markets. Rats were obtained from the six districts of Malang i.e. Koljen, Lowokwaru, Sukun, Kedungkandang, Blimbing, and Wagir.

2.3 Data collection, and sample collection

Rat collection was carried out from August to October 2021 using live traps. All specimens were euthanized according to the standard procedure followed by blood and peritoneal fluid collection (AVMA: 2020 edition). Morphometric measurements of rats were performed with unit length in millimeters (mm) and weight in grams. Rats' species were determined based on specific characteristics. The standard identification parameters of rats included the total length of body and tail (Total Length), length of tail, hindfoot length, ear length, weight measurement, and added head length measurement (Yuliadi and Muhidin 2016).

2.4 Parasites identification

Blood and peritoneal fluid were smeared on the glass slides and stained using Giemsa staining solution. The prepared smear was observed under 100x microscopes Olympus CX-23 (Olympus Corporation, Japan) with oil emersion and captured by OptiLab Advanced Plus camera (PT Miconos, Indonesia). Spotted parasites were measured using *ImageJ*. According to Desquesnes et al. (2002), *T. lewisi* has konetoplast in the sub-terminal region and a long slight posterior end. The nucleus of this hemo-protozoan was in the anterior region and equipped with free flagella. Montoya et al. (2015) guideline was used for the *T. gondii* morphology study and found that its tachyzoite is crescentic or oval shape and has 5 to 7 length and 2 to 3 µm width. The anterior has a cone-shaped conoid structure with a round posterior end. Dense granules are spread throughout the cytoplasm. The single nucleus is located toward the central area of the cell or the hind end (Vismara et al. 2021).

2.5 Geographical Information System on Parasite

Geographical Information Systems (GIS) maps were created using the QGIS 3.4.5 Madeira. The maps were made by adding the coordinates of the sampling points on the layers of geographic and administrative shapefiles. Shapefiles of the administrative boundaries and detailed bodies of water were derived from the Digital Elevation Model National (DEMNAS) Indonesia. The elevation shapefile was derived from the 50 m Digital Elevation Model database.

2.6 Statistical analysis

Descriptive analyses were used to describe the morphology and morphometry of *T. lewisi* and *T. gondii*. In addition, proportions of protozoan infection were using IBM SPSS (Statistic Package for Social Sciences) 27 version (IBM Corp., Armonk, New York). The Chi-square tests were performed to compare *Trypanosoma* and *Toxoplasma* infection proportions per genus, age, and gender. Risk analyses were conducted using the Odds Ratio test (OR) (Allam et al. 2019).

3 Results

3.1 Identification of Rats species

Total 50 rats were caught and processed for subsequent tests. The results suggested that 10 (20%) rats were positive for blood protozoa and 1 (2%) for tissue protozoa among all collected specimens. Based on quantitative identification of rats (Table 1), all *Rattus rattus* have 39-210 grams body weight, 200-380 mm total length, 70-190 mm tail length, 25-35 mm hindfoot length, 30-55 mm head length, and 10-23 mm ear length. Further, *R. norvegicus* have a more extensive body and have 63-528 grams body weight, 300-560 mm total size, 125-240 mm tail length, 30-45 mm hind foot length, 40-70 mm head length, and 10-25 mm ear length. The specimens of *Mus musculus* have 19-30 grams body weight, 180-220 mm total length, 55-125 mm tail length, 25-35 mm hind foot length, 30-40 mm head length, and 15-20 mm ear length. These results are in agreement with the data reported by Yuliadi and Muhidin (2016), who suggested that *R. rattus* is a medium-sized rat with a total length of 170-370 mm and have a nipple formula 2 + 3. Meanwhile, the *R. norvegicus* is a giant rat with a length of 190-550 mm with a nipple formula. 3 + 3, whereas *M. musculus* is small-sized with a total length of no more than 180 mm and nipple formula 3 + 2.

3.2 Morphology and Morphometry of *T. lewisi*

Microscopic observation revealed that *T. lewisi* showed a leaf-like shape, a long slight posterior end with an oval-shaped kinetoplast located sub-terminal, and a nucleus situated in the anterior part. They are equipped with the free flagellum and undulating membrane (Figure 1). Based on the results of the morphometric examination of *T. lewisi* in specimens, the body length of *Trypanosoma* ranges from 13.36-37.19µm, with an average of 29.26 µm. The body widths were about 1.11 -2.38 µm, with an average of 1,783µm. The visible nuclei were measured 1.41-3.19 µm on average 2.16 µm, and the nucleus widths were around 1.12 – 1.56 µm with an average of 1.27 µm. Further, the length of the kinetoplast raged from 0.32 – 1.19 µm, with an average of 1.08µm. The distances from the nucleus to the kinetoplast were about 4.55 – 8.61 µm with an average of 6.79 µm. Moreover, the free flagellum length was 6.88 – 9.02 µm with 8.11 µmon average (Table 2).

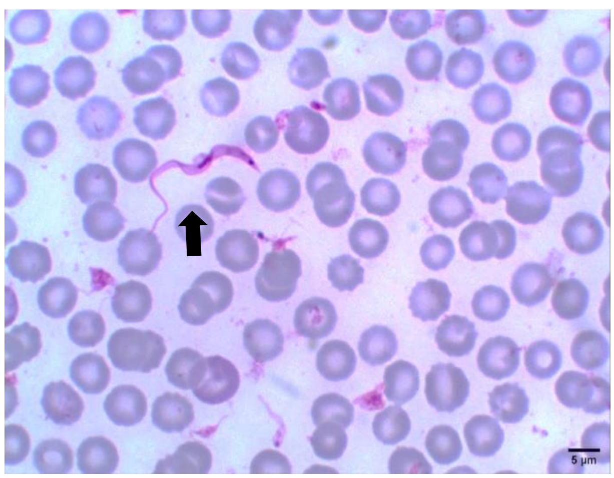

Figure 1 *Trypanosoma lewisi* (arrow) in rat blood, (Giemsa stained; Magnification 100x objective), Scale bar: 5 µm

Table 1 Morphometric recording of rats captured in Malang city

Species of Rats	Morphometric Measure						Nipple Formula
	body weight (grams)	total length (mm)	tail length (mm)	hindfoot length (mm)	head length (mm)	ear length (mm)	
Rattus rattus	39-210	200-380	70-190	25-35	30-55	10-23	2 + 3
Rattus norvegicus	63-528	300-560	125-240	30-45	40-70	10-25	3 + 3
Mus musculus	19-30	180-220	55-125	25-35	30-40	15-20	3 + 2

Table 2 The morphometric measure of trypanosomes in wild rats

Morphometric	*T. lewisi* (µm) In this study	*T. lewisi* (µm) (Sarataphan et al., 2007)	*T. evansi* (Sarataphan et.al, 2007)
Length	13.36-37.19	31.8 ± 6.4	21.0 ± 0.6
Widths	1.11 -2.38	2.7 ± 3.4	1.5 ± 0.3
Distances from the nucleus to kinetoplast	4.55 – 8.61	7.8 ± 1.3	6.1 ± 0.1
Flagellum	6.88 – 9.02	7.8 ± 4.5	4.2 ± 1.1

Table 3 Proportion of Parasite in rats according to the sex of rats

Parasite	Male (n= 27)	Female (n= 14)	T.P (%)
	Positive (%)	Positive (%)	
Trypanosoma lewisi	6 (12%)	4 (8%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	7 (14%)	4 (8%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion

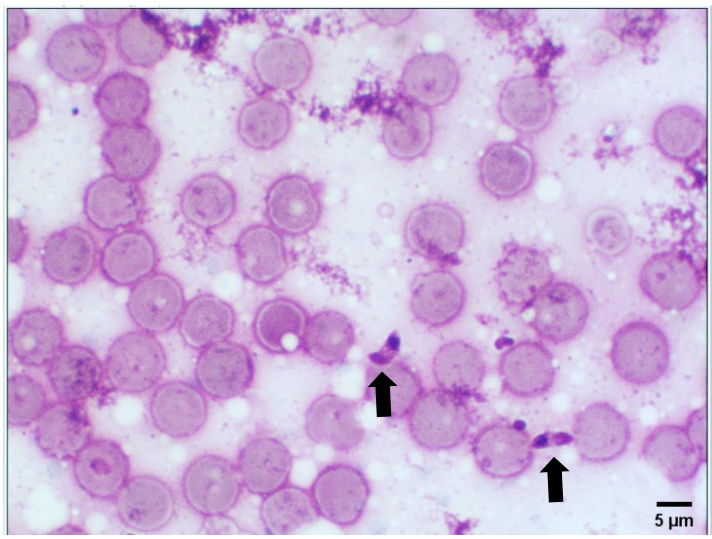

Figure 2 Tachyzoites in image smear of peritoneal fluid, here Note: semi-circular shaped of tachyzoites stadium (arrows), compared with host red blood cells; Giemsa stain. Scale bar: 5 µm

3.3 Morphology and Morphometry of *T. gondii*

The tachyzoite was oval to crescentic shapes and the nucleus was located in the posterior part. The morphometry of *T. gondii* length from posterior to anterior end ranged from 5.67 to 6.83 µm with an average of 6.226 µm. The width ranged from 1.48 to 2.70 µm with an average of 2.091 µm. The visible nuclei were measured 1.44-2.46 µm in length with 1.86 µm in middle, and the nucleus width was around 1.13 – 1.57 µm, with an average of 1.326 µm (Figure 2).

3.4 The proportion of *T. lewisi* and *T. gondii* infection in Rats

Based on the blood smear examination of 50 rats from August to October 2021, the proportion of *T. lewisi* infection was recorded at 20%, while it was recorded at only 2% in the case of *T. gondii*. The results of blood smear examination on rat samples showed that six male rats were positive, and three female rats were infected with *T. lewisi*. In addition, a positive specimen infected with *T. gondii* in the tachyzoite stage based on a peritoneal fluid smear is a male (Table 3). The data obtained were tested using the Chi-square test resulting in the Asymp value at 0.963 (> 0.05). There was no significant correlation between the proportion of infection and rat sex. Furthermore, the Odds Ratio (OR) test was implemented and presented a 0.964 OR score (Cl 95%). This result indicates that gender increases the infection risk at 0.964 times the proportion of the incidence of protozoa infection. Meanwhile, based on the Relative Risk (RR) test results it was found that male rats had a 0.994 times chance (Cl 95%) to be not infected. In contrast, female rats had a 1.030 chance of not being infected with *T. lewisi* and *T. gondii.*

Table 4 Proportion of Parasite in rats according to the genus of rats

Parasite	Genus Rattus (n= 45)	Genus Mus (n= 5)	T.P (%)
	Positive (%)	No Positive (%)	
Trypanosoma lewisi	9 (18%)	1 (2%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	10 (20%)	1 (2%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion.

Table 5 The proportion of Parasite in rats according to the age of rats

Parasite	Adults (n= 38)	Juvenile (n= 12)	T.P (%)
	Positive (%)	Positive (%)	
Trypanosoma lewisi	8 (16%)	2 (4%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	9 (18%)	2 (4%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion.

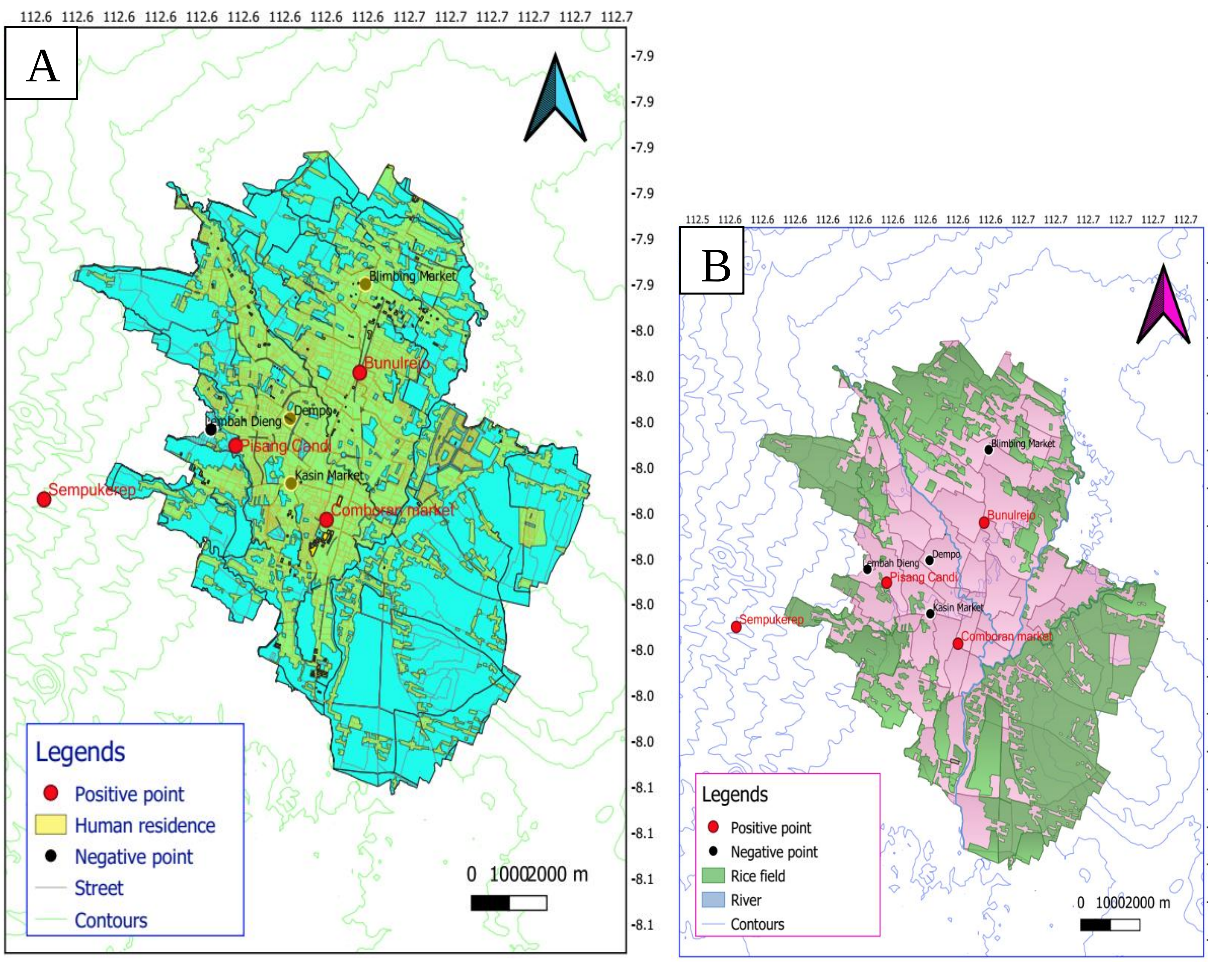

Figure 3 The map shows the distribution of Protozoa in wild rats in Malang East Java; (A) Modern map with human residence and street layers, and (B) Natural map with rice field and river layers.

The data obtained were tested by chi-square and the Asymp value was reported as 0.546 (> 0.05), concluding there was no significant relationship or correlation between the genus of rat and the proportion of infection. Furthermore, the Odds Ratio (OR) test was implemented and the OR value was 2.43 (Cl 95%). This result means that the genus increases the risk by 0.96 times the proportion of the incidence of protozoan infection. Meanwhile, based on the Relative Risk (RR) test results, it was found that genus *Rattus* had a 0.85 times chance (Cl 95%) were not infected, and genus *Mus* had a 1.91 times chance of not being infected with the protozoal disease (Table 4).

Based on the age of the caught wild rats, the study results obtained eight adult rats infected with protozoal diseases, and among these two were positive (Table 5). The data of this study was accomplished by the Fisher's Exact Test. The Exact Sig value was 0.23 because the Exact Sig value was > 0.05, there was no significant correlation between the age of the rat and the proportion of cases of protozoa infection. Furthermore, the Odds Ratio (OR) test was carried out to determine the relationship between protozoal and rat age, and the obtained result was 0.31 (Cl 95%). Meanwhile, based on the results of the Relative Risk (RR) test, it was found that adult rats (adult) had 0.77 times (Cl 95%) chance of not being infected by *T. lewisi* or *T. gondii*. Moreover, young (juvenile) rats had 2.34 times (Cl 95%) chance of not being infected by hemo-protozoan or tissue protozoa.

3.5 Geographical Distribution of Parasites

Based on the results of blood tests, it was found that ten rats were positive for *T. lewisi*, and a specimen was positive for *T. gondii*. Among the infected rats, five rats were reported from the Sempukerep, one rat from Pisang Candi Sukun District, three from the Comboran market Klojen District, and one from Bunulrejo Belimbing District Malang. Moreover, specimen positive *T. gondii* was recorded in Comboran Market Klojen District. Based on these results, a map of the distribution of the blood protozoan *T. lewisi* and *T. Gondii* were illustrated in Figure 3. Through regional distribution, it was found that 5(50%) specimens were positive for *T. lewisi* in Wagir District. In addition, there were 3 (30%) rats infected in the Klojen district. Furthermore, in Sukun and Blimbing Districts only 1 infected rat was found infected from each district (10% from each district).

4 Discussion and conclusion

Blood protozoa, *T. lewisi* is a trypanosome found in rodents and *Xenopsylla cheopis* fleas have a role as vectors. The study chose the dry season months from August to October for sampling. In this season, *Xenopsylla cheopis* often suck blood from animal hosts for egg development, thus potentially spreading *T. lewisi* infection (Hadi and Soviana 2018).

The morphology seen from the blood smear method is the same as Desquesnes et al. (2002). In contrast to *T. evansi* and *T. cruzi*, some differences have been observed in *T. lewisi*, it has a subterminal kinetoplast, a sharp posterior end, a centrally located nucleus, a regular wave shape undulating membrane, and free flagella (Teixeira et al. 2011; Maharani 2016). While in the case of *T. evansi* and *T. cruzi*, the location of the nucleus is in the central part and the undulating membrane has an irregular shape (Truc et al. 2013).

Along with this, in the morphometry description also, *T. lewisi* has some differences as compared to *T. evansi* and *T. cruzi*. The morphometry of *T. lewisi* described in this study is similar to Rabieel et al. (2018) description. *T. lewisi* has a larger size as compared to *T. evansi* and *T. cruzi*, it has an average length of 31 - 44 µm and a width of 1.9 – 3.2 µm while in the case of *T. evansi* length is 15 – 34 µm, 3.15 µm wide, nucleus length 8.21 µm, nucleus width 3.10 µm, kinetoplast length 5.16 µm, undulating membrane length 12.77 µm, and the distance from the nucleus to kinetoplast is 14.8 µm, and similar size was reported for the *T. cruzi* (Levine, 1985; Sarataphan et al. 2007).

T. gondii infection in rats is another important parasite of public health and the veterinary field. Rats potentially transmitted diseases directly or indirectly to humans through contaminated food or water or via an intermediate host (Paramasvaran et al. 2009). The intraperitoneal fluid smear revealed the stadium of tachyzoite *T. gondii*, a parasite of zoonotic importance, with a proportion number of 2%. This finding aligns with studies in Malaysia, which reported a low proportion (6.7%) of *T. gondii* infection in rats (Tijjani et al. 2020). The parasite in rats could be linked with the presence of the stray cat in Malang and poor sanitary conditions in the sampling area. The climatic and environmental conditions in the study area (humidity and tropical situation) influence the persistence and spread of *T. gondii* oocyst. Rats may not transmit toxoplasmosis to a human directly. However, they can be intermediate hosts or serve as a source of infection in cats in this study area. This becomes a source of infection to humans via infective oocysts and consuming poorly cooked meat tissue cysts (Vujanic et al. 2011).

In terms of the proportion of disease incidence, according to Alias et al. (2014), male rats have a greater chance of being infected with blood or tissue protozoa because male rats can explore a larger area than female rats. This allows the broader transmission of protozoan infection. This is in the agreement with this study, where 6 male and 4 female rats were found positive for protozoa. Further, an analysis of the proportion of disease based on the age of the rats was also carried out. The obtained results suggested that among the caught rats, eight adult rats, and two young (juvenile) rats were recorded positive for protozoa. This is in line with the research of Novita (2019) who reported lesser infection in the young rats (juvenile) and this might be due to the lighter body weight as compared to the adult rats, these researchers reported a correlation between parasite densities with rat body weight. Young rats have a lighter body weight due to the number or density of parasites being more diminutive than adult rats. Therefore, young rats have a lower chance of being infected with blood or tissue protozoan.

Making the map is intended to determine the magnitude of the risk factors for protozoal infection. Based on the mapping, it was found that residential areas, markets, and urban areas are highly prone to infection with protozoa diseases because there are very abundant food sources for rats in these areas. The geographical map presented in figure 3 (A and B) is of Malang. The risk and potential of *T. lewisi* and *T. gondii* spreading were reported in several areas i.e. Sempukerep, Pisang Candi, Bunulrejo, and Comboran markets. The layers shown in map A showed modern layers such as human residences and streets. While map B showed natural layers such as rivers and rice fields. Rats generally live near rivers, water sources, human residences, or urban areas. Hence rats potentially spread the protozoan to animals or humans living near this area. In figure 3, the location of the positive rat sampling is symbolized in the form of a red dot (dot), based on the coordinates of the sampling point, while the black dot represents the negative sampling area. The rapid developments of human residence and agriculture have caused a gradual increase in food products and provided decent live places in urban areas for populations of rats (Khaghani 2007).

In most cases, rats are a significant reservoir for diverse parasites with potential zoonotic diseases (Seifollahi et al. 2016). The data showed that 20% of the exanimate rats were found positive. Further, it was reported that rats caught in the sampling area were infected with two protozoa species, and both have public health concerns. This pushes up a potential risk of disease carried by rodents to humans in Malang. However, research for a better understanding of the dangerous exposure places is needed. The awareness promotion on control and prevention of the rodents population is highly required in most communities that live in close contact with rats. Though the clinical significance to human and public health impact is questionable, further study and surveillance of rodent-borne parasitic pathogens will provide more information for pre-emptive action. In a nutshell, public health is significant in Malang East Java, which can cause infection of zoonotic protozoal in humans, such as *Trypanosomes* and *Toxoplasmosis*. Therefore, it is crucial to take into consideration the role of rats in spreading infectious or parasite diseases in Malang for better control.

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