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Molecular detection of *Cryptosporidium* spp. among wild rats in Surabaya, East Java, Indonesia

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Abstract

Background: Humans and animals who have an acute case of diarrhea can be infected with *Cryptosporidium* spp. Within the category of water-borne disease, it is a zoonotic disease. The zoonotic disease *Cryptosporidium* is among the several pathogens carried by wild rats (*Rattus* spp.). The risk of spreading this disease is rather significant in urban environments because rats are often close to people.

Aim: This study aims to detect Cryptosporidium spp. infection in wild rats in Surabaya, East Java, Indonesia.

Methods: Through necropsy, a total of 100 wild rats' intestines were sampled for feces. Microscopic observation of the presence of *Cryptosporidium* was carried out using the float test with a combination of Ziehl Neelsen (ZN) staining. Molecular detection of *Cryptosporidium* spp. positive results used the *Cryptosporidium* oocyst wall protein (COWP) gene with polimerase chain reaction method.

Results: The results showed that 69 samples were positive for containing *Cryptosporidium* spp. oocysts and with ZN staining to confirm the diagnosis, the staining results showed *Cryptosporidium* spp. oocysts dark pink with a clear cavity inside with a percentage of 95.65% in *Rattus norvegicus* and 61.03% in *Rattus tanezumi*. In residential and densely populated environments the percentage of *Cryptosporidium* spp. amounted to 66.66% and in the market environment amounted to 74.19%. The percentage of *Cryptosporidium* spp. in the North Surabaya region was 42.85%, South Surabaya 100%, West Surabaya 37.5%, East Surabaya 81.39%, and Central Surabaya 65.38%. Molecular detection of *Cryptosporidium* spp. positive results were obtained using the COWP gene 550 bp.

Conclusion: This study aims to detect Cryptosporidium spp. infection in wild rats in Surabaya, East Java, Indonesia. The high number of cases of cryptosporidiosis in wild rats has the potential to be a reservoir for the spread of the disease. The Cryptosporidium spp can detected with COWP in 550 bp in wild rats in Surabaya, East Java, Indonesia.

Keywords: COWP gene, *Cryptosporidium* spp., Wild rat, Ziehl-Neelsen, Zoonosis.

Introduction

Cryptosporidiosis is a zoonotic disease and is classified as a waterborne disease, that is transmitted between living creatures due to contamination in the form of microorganisms or dangerous substances through water (Mufasirin *et al.*, 2020). More than 40 species and genotypes of *Cryptosporidium* have been identified worldwide (Zahedi *et al.*, 2021). In rodents, a total of 21 species and 21 genotypes of *Cryptosporidium* were found (Zhao *et al.*, 2019). The *Cryptosporidium* species most frequently detected in wild rats are *Cryptosporidium parvum* and *Cryptosporidium muris* (Koehler *et al.*, 2018).

The infective form of *Cryptosporidium* spp. are oocysts excreted in the feces of infected hospes. The clinical signs and symptoms of cryptosporidiosis are contingent upon the immunological condition of the individual. In immunocompetent individuals, infection with *Cryptosporidium* spp. can be asymptomatic or with symptoms of acute diarrhea that can heal itself. Meanwhile, in immunocompromised individuals, diarrhea can become chronic and can even cause death (Roy *et al.*, 2004). The prevalence of *Cryptosporidium* spp. in animals varies greatly, in rodents as much as 22% in Europe (Wijayanti, 2017), France in 2021 at 2.1%–63.0% (Livia *et al.*, 2021), China at 8.2% (Zhao *et al.*, 2015), and Japan at 8% using the immunofluorescence assay (IFA) technique and 38% positive with the Nested polimerase chain reaction (PCR) technique (Kimura *et al.*, 2007). This research

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aims to detect *Cryptosporidium* infection in wild rats in Surabaya parasitologically and molecularly. The purpose of this study is to identify whether wild rats harbor the protozoan *Cryptosporidium* spp. The broad range and high incidence of *Cryptosporidium* spp. in wild rodents contribute significantly to the spread of the disease to humans and have the potential to become a significant source of contaminated water.

Materials and Methods

Sampling technique

The study was carried out in 2023 from June to October. Surabaya, East Java, Indonesia, was the site of the sample. The samples were examined under a microscope at Universitas Airlangga's Faculty of Veterinary Medicine's Laboratory of Veterinary Parasitology. The Institute of Tropical Disease at Universitas Airlangga carried out PCR testing. Under Airlangga University's 2.KEH.080.07.2022, the Animal Care and Use Committee of the Faculty of Veterinary Medicine authorized the experimental procedure.

Wild rat samples are collected in five Surabaya areas (North, West, South, East, and Central of Surabaya) as well as in each area where sampling is done including the communities, the market environment, and the densely populated housing environment habitation. The Lemeshow formula was utilized to calculate the sample in this investigation (Lemeshow *et al.*, 1990) in a state of population unknown. The following computations were made:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

n = Number of samples
$Z = z$ distribution value at 95% CI
P = Maximum probability estimate
d = 10% accuracy

$$n = \frac{Z^2 P(1-P)}{d^2}$$

$$n = \frac{1960^2\ 0.5(1-0.5)}{0.1^2}$$

$$n = \frac{3.8416\ 0.25}{0.01}$$

$$n = 96.04$$

$$n = 96$$

The result of the calculation of the formula Lemeshow obtained the sample number result. The minimum required in this study is 96 samples, the total number of samples. In this study, there were 100 wild rats.

Parasitological and Ziehl Neelsen (ZN) method

Fecal samples were taken from the digestive tract of rats by necropsy, then the feces were examined using the sugar flotation centrifugation method. ZN staining technique was applied on smears. After the smear has dried and been fixed with a Bunsen burner, carbol fushin is applied to the slide and heated until it turns to steam. The slide is then rinsed with water and, depending on the smear's thickness, decolored for 1 minute with 2.5% sulfuric acid. The slide is then stained once more by dripping 1% methylene blue for 1 minute, dried, and examined at 400× or 1,000× magnification (Tahvildar and Salehi, 2014).

Polymerase chain reaction

Extraction by sonication process first and followed by the Qiagen® kit. The primer design uses the *Cryptosporidium* oocyst wall protein gene (COWP) which is found in all species of *Cryptosporidium* spp. COWP primers: forward primer 5′-GTAGATAATGGAAGAGATTGTG-3′ and reverse primer 5′-GGACTGAAATACAGGCATTATC TTG-3′ with 550 bp. The steps involved in PCR amplification are as follows: 3 minutes of initial denaturation at 94°C; 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, 60 seconds of extension at 72°C, and 7 minutes of elongation at 72°C. Electrophoresis is performed after PCR findings (Spano *et al.*, 1997).

Ethical approval

The Animal Care and Use Committee of the Faculty of Veterinary Medicine at Airlangga University, with approval number 2.KEH.080.07.2022, authorized the experimental procedure.

Results

Parasitological test

Microscopic examination of rat feces samples in Surabaya using the floating method aimed at detecting *Cryptosporidium* spp. oocysts. The results showed that 69 out of 100 samples contained *Cryptosporidium* spp. oocysts. The oocysts found were round in shape with a diameter of 3.67 µm (Fig. 1). This is in accordance with the report by Mufasirin *et al.* (2020) in their research that the size of the oocysts of *Cryptosporidium* spp. 2–6 µm and has a round shape with four sporoozytes inside.

ZN method

Feces examination techniques can be combined with ZN staining to confirm the diagnosis, the staining results show *Cryptosporidium* spp oocysts. round, dark pink in color with a clear cavity inside (Fig. 2). This is in accordance with the report by Jassim and Al-Mussawi (2021) in their research that the oocysts of *Cryptosporidium* spp. will be pink in color and is in accordance with the research of Rekha *et al.* (2016) that with ZN staining of *Cryptosporidium* spp oocysts. It appears dark pink in color with a clear cavity inside.

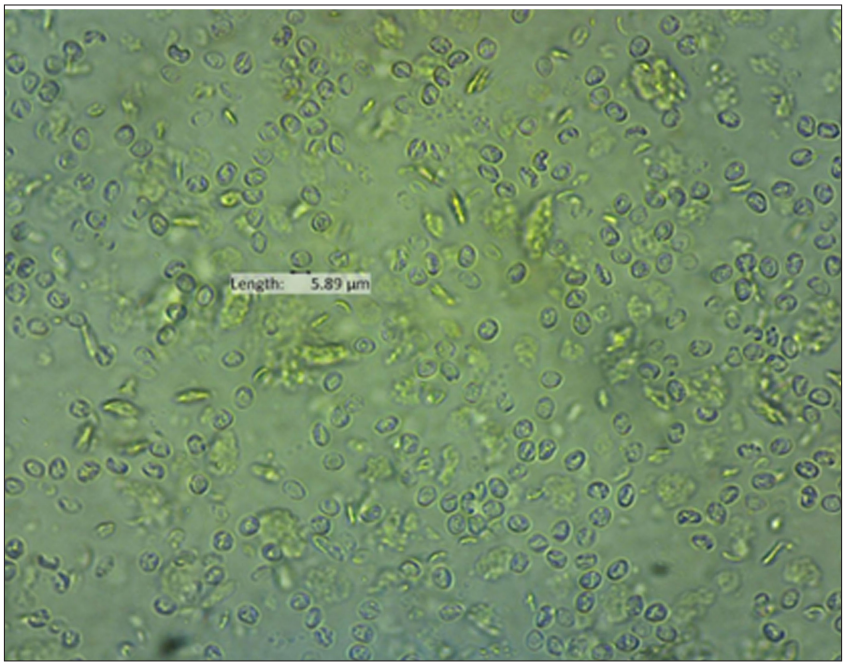

Fig. 1. Oocysts of *Cryptosporidium* spp.

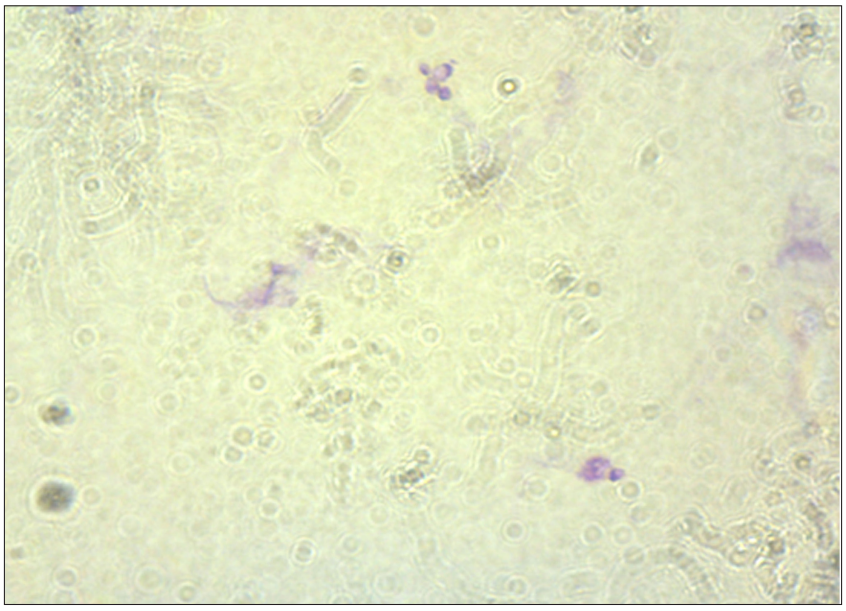

Fig. 2. Oocysts of *Cryptosporidium* spp. with ZN staining.

Prevalence Cryptosporidium spp of wild rats in Surabaya

The prevalence in parasitological tests is the percentage of rats infected with *Cryptosporidium* spp. compared to the total of sample. The prevalence rate is calculated using:

$$\text{Prevalence} = \frac{\text{number of cases}}{\text{total of sample}} \times 100$$

The results showed that the prevalence of *Cryptosporidium* spp. oocysts were 69% (69/100). Prevalence of *Cryptosporidium* spp. oocysts in *Rattus norvegicus* were 95.65% (22/23) and 61.03% (47/77) in *Rattus tanezumi*. Prevalence of *Cryptosporidium*

spp. oocysts in wild rats in residential were 66.66% (22/33), in densely populated environments, were 66.66% (24/36), and in the market environment 74.19% (23/31). Prevalence of *Cryptosporidium* spp. in wild rats in the North Surabaya region, it was 42.85% (3/7), South Surabaya 100% (8/8), West Surabaya 37.5% (6/16), East Surabaya 81.39% (35/43), and Central Surabaya 65.38% (17/26) (Table 1). Molecular detection of *Cryptosporidium* spp. positive results were obtained using the COWP gene with PCR method.

Molecular detection of Cryptosporidium spp. in wild rats

Data from the results of microscopic tests and molecular tests were analyzed qualitatively and explained descriptively. Molecular analysis by a number of amplified band fragments is based on research by Spano *et al.* (1997), there are 34, 106, 125, and 285 bp (Fig. 3).

Molecular testing with PCR aims to confirm positive results for *Cryptosporidium* spp. after microscopic examination. Detection of *Cryptosporidium* spp. in this study used the COWP gene with a band of 550 bp with fragments of 34, 106, 125, and 285 bp (Spano *et al.*, 1997). The results of the research show visualization of the PCR product using the COWP gene, amplification of a DNA band of 285 bp with an annealing temperature of 52.6°C with the DNA preextraction method using the heating method, and amplification of a DNA band of 106 bp temperature annealing 48°C DNA preextraction method using the sonication. PCR results can be seen in Figures 4 and 5.

The results detection of *Cryptosporidium* spp. on wild rats in Surabaya using the PCR method, positive results were obtained 285 bp was amplified with an annealing temperature at 52.6°C and 106 bp with an annealing temperature at 48°C using the COWP gene primer. The results obtained are only two bands appeared out of the four bands that should appear in the PCR product; 34, 106, 125, and 285 bp (Spano *et al.*, 1997).

Table 1. Demographic data for prevalence toxoplasmosis of wild rats in Surabaya.

Category	Variables	Prevalence (%)
Subdistrict	West Surabaya	37.5% (6/16)
	Central Surabaya	65.38% (17/26)
	South Surabaya	100% (8/8)
	East Surabaya	81.39% (35/43)
	North Surabaya	42.85% (3/7)
Habitat	Residential	66.66% (22/33)
	Densely populated	66.66% (24/36)
	Markets	74.19% (23/31)
Species	*Rattus tanezumi*	61.03% (47/77)
	Rattus norvegicus	95.65% (22/23)

Discussion

Detection of *Cryptosporidium* spp. in this study used the COWP gene with a band of 550 bp with fragments of 34, 106, 125, and 285 bp (Spano *et al.*, 1997). The only known apicomplexan oocyst membrane protein is the *Cryptosporidium* oocyst wall protein 1 (COWP1) gene, which codes for the COWP. COWP1 is found in the wall-forming bodies of mature macrogametes as well as the inner oocyst wall, according to immunoelectron microscopy results (Templeton *et al.*, 2004). The COWP gene PCR has a specificity value of 99.6% and a sensitivity value of 90% (Weinreich *et al.*, 2021).

Cryptosporidium is one of the numerous zoonotic infections carried by wild rats (*Rattus* spp.). There is a significant chance that this disease will spread because rats and people live close together in an urban environment (Zhao *et al.*, 2019). One possible source of infection is rodents. There may also be mechanical transmission by insects, birds, and people (Martin and Aitken, 2000). Season and the quantity of rats in the community are additional risk variables. High levels of environmental contamination are ensured by the enormous number of oocysts released upon infection (Dixon *et al.*, 2011).

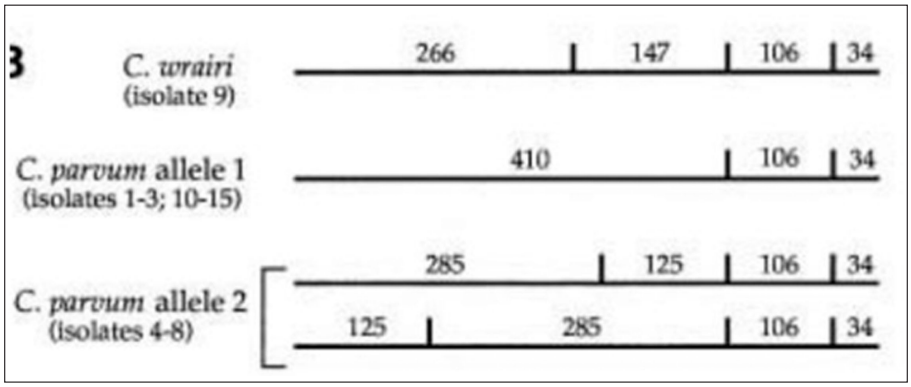

Fig. 3. Length of amplified band fragments (Spano *et al.*, 1997).

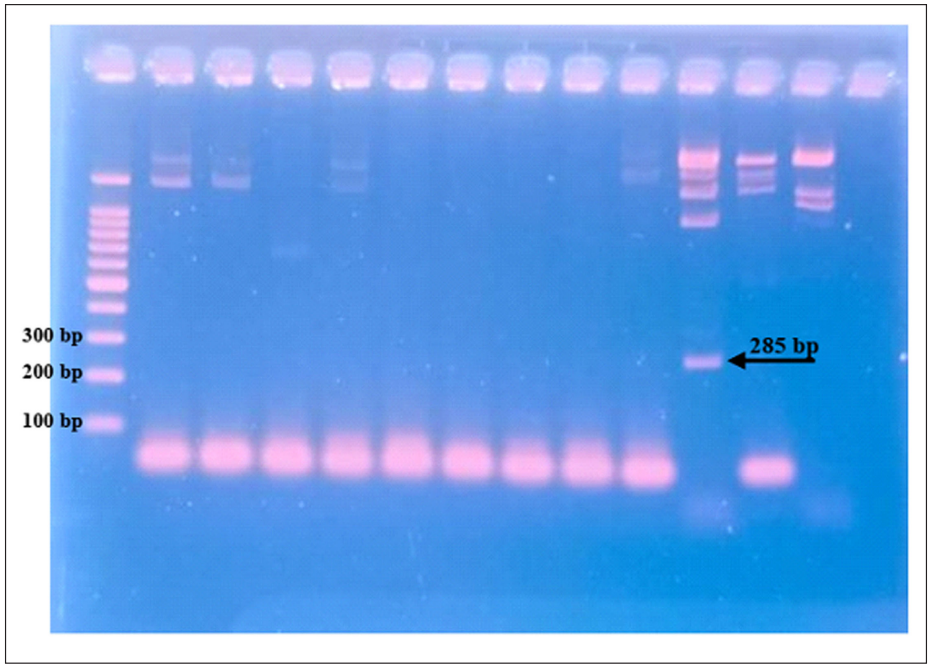

Fig. 4. PCR results of the COWP gene 285 bp.

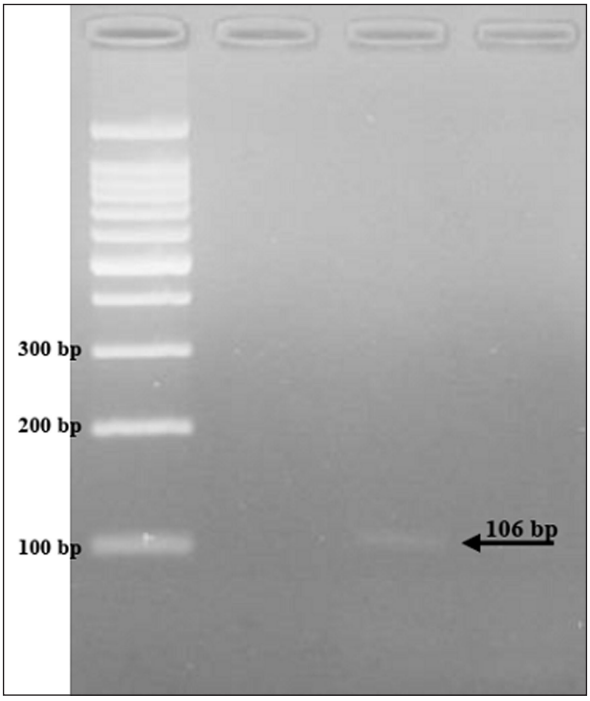

Fig. 5. PCR results of the COWP gene 106 bp.

When the infectious oocyst is consumed by the new host, infection results. The upper gastrointestinal tract becomes excited after consumption. When oocysts enter the digestive system, they produce infectious sporozoites, or trophozoites, which cling to intestinal epithelial cells (Bouzid *et al.*, 2018). In a host that is vulnerable, one oocyst is enough to cause infection and illness. The fecal-oral pathway is the means by which environmentally resistant oocysts spread (Smith *et al.*, 2007). Because of the high likelihood that *Cryptosporidium* oocysts in soil may find their way into water supplies, populations residing close to rivers should be viewed as possible sources of waterborne disease (Ghazy *et al.*, 2015).

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Conflict of interest

There is no conflict of interest, according to the authors.

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Authors' contributions

ES coordinated the research, authored the article manuscripts, reviewed the literature, and designed and conceptualized the proposal. PH, BS, PG MM, HP, ENI, PH Rearing and trapping wild rats, gathering information, reviewing the literature, and analyzing the information; SK also worked with flow cytometry and data processing. Each author edited and proofread the work.

Data availability

The manuscript contains all the data needed to support the study's conclusions; no other data sources are needed.

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