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Species diversity of *Entamoeba* and gastrointestinal parasites as co-infection in pigs in Kupang, East Nusa Tenggara, Indonesia

DONY CHRISMANTO[1,6], NUNUK DYAH RETNO LASTUTI[2,3,♥], LUCIA TRI SUWANTI[2], POEDJI HASTUTIEK[2], ENDANG SUPRIHATI[2], DYAH AYU KURNIAWATI[4], AJI WINARSO[5]

[1]Program of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Kampus C Mulyorejo, Surabaya 60115, East Java, Indonesia

[2]Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Kampus C Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel./fax.: +62-031-5993015, ♥email: nunukdyah53@gmail.com

[3]Postgraduate School, Universitas Airlangga. Jl. Airlangga No. 4-6, Surabaya 60115, East Java, Indonesia

[4]Indonesian Research Center for Veterinary Sciences, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture Republic Indonesia. Jl. Harsono RM No.3, Pasar Minggu, South Jakarta 12550, Jakarta, Indonesia

[5]Laboratory of Parasitology, Department of Animal Disease Sciences and Veterinary Public Health, Faculty of Medicine and Veterinary Medicine, Universitas Nusa Cendana. Lasiana, Klp. Lima, Kupang, Indonesia

[6]Department of Health, Faculty of Vocational, Universitas Airlangga, Jl. Airlangga No. 4-6, Surabaya 60115, East Java, Indonesia

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Abstract. *Chrismanto D, Lastuti NDR, Suwanti LT, Hastutiek P, Suprihati E, Kurniawati DA, Winarso A. 2023. Species diversity of* Entamoeba *and gastrointestinal parasites as co-infection in pigs in Kupang, East Nusa Tenggara, Indonesia. Biodiversitas 24: 233-240.* Protozoa *Entamoeba* spp. is the causative agent of amoebiasis that can infect humans and livestock. Several species that infect pigs are *E. suis*, *E. polecki*, *E. histolytica*, and *E. coli*. However, microscopic examination through morphological identification of parasites couldn't determine the genus and species level identity, leading to misdiagnosis. Therefore, molecular techniques are needed for accurate diagnosis with species-specific primers. In this study, 100 fecal samples were collected from pig farms in Kupang, East Nusa Tenggara Province, Indonesia. This study aims to identify, using molecular techniques, the presence of *Entamoeba* spp. with the SSUrRNA gene as a marker to determine the diversity of *Entamoeba* species in pigs and to detect co-infection with other gastrointestinal parasites in Kupang. The result was found: *E. suis* 98% (98/100), *E. polecki* 44% (44/100), *E. polecki* ST3 10% (10/100), and *E. coli* 20% (30/100) spread on pig farms in 8 villages in Kupang. Furthermore, *E. polecki* ST3 and *E. coli* finding show that pigs in several areas in Kupang are infected with potentially zoonotic species. In addition to finding various species of *Entamoeba* by Polymerase Chain Reaction (PCR) amplification, based on microscopic examination, other gastrointestinal parasites were also found as co-infections, namely: *Eimeria* sp. (34%), *Cystoisospora suis* (30%), *Strongyloides* sp. (34%), *Oesophagostomum* sp. (51%), *Metastrongylus* sp. (18%), and *Trichuris suis* (8%). That is the first report concerning mixed infections with *E. suis*, *E. polecki* ST3, *E. coli,* and other gastrointestinal parasites in pigs in Kupang, Indonesia.

Keywords: *Entamoeba* spp., Gastrointestinal parasites, SSUrRNA, *Suis* sp.

INTRODUCTION

Several species of porcine *Entamoeba*, *E. suis*, *E. polecki* (subtype 1 and 3), *E. coli,* and *E. histolytica* have been detected in a few countries by molecular methods (Matsubayashi et al. 2015; Ji et al. 2019; Wardhana et al. 2020). Gastrointestinal parasites, including *Entamoeba*, are generally transmitted through contaminated drinking water or food with mature cysts excreted in the feces (Kantor et al. 2018). *Entamoeba* spp. has also been detected in moist soil around drinking water sources (Hirashima et al. 2017). Cyst and trophozoite stages of *Entamoeba* can be found in pig manure, which is related to the parasite's life cycle in its host. However, the microscopic examination has difficulties distinguishing species determination (Matsubayashi et al. 2016). In the microscopic examination, species were determined based on the size of the cyst, the number of nuclei, and the presence of a ribosome crystal arrangement (Matsubayashi et al. 2016; Stensvold et al. 2018). The incidence of gastrointestinal parasites caused by protozoa or helminths is frequently found in pigs. The main effects are loss of appetite, decreased daily nutrition, decreased feed efficiency, and the potential for infection with other pathogens (Carrero et al. 2020). Protozoa and helminths are parasites that can infect the digestive tract of pigs; some are zoonotic, namely *Ascaris* sp., *Trichuris* sp., *Balantidium coli*, *Entamoeba* sp., and *Blastocystis* sp. (Ahmed et al. 2020; Widisuputri et al. 2020). Furthermore, the potential risk factors for the prevalence of *Entamoeba* spp. are hygiene, socioeconomic, exposure to human and animal waste, and household wastewater (Carrero et al. 2020). In the study conducted by Ji et al. (2019), a potentially zoonotic *Entamoeba* species was found in China (*E. polecki* ST1 and *E. polecki* ST3); this needs to be considered due to the high population density of pigs in China is exposure to zoonotic risk that cannot be avoided.

Research results have reported risk factors associated with the prevalence of *E. histolytica* in three ethnic groups in Malaysia, showing a high prevalence in people of eatinghabits with their hands, consuming raw vegetables, and have frequent contact with animals (Shahrul et al. 2012).

Pigs infected with *Entamoeba* spp. clinical symptoms such as diarrhea and, on pathological examination, showed swelling of the small intestine, bleeding, ulcers, and necrotic events occurred as well as the presence of inflammatory cells in the lamina propria layer and mucosal tissue (Matsubayashi et al. 2019). In addition, histopathological changes indicated the presence of trophozoite infiltration in the colon (Komatsu et al. 2019; Wardhana et al. 2020). In 2014, researchers reported *E. histolytica* was pathogenic in pig farms and had zoonotic problems. Others research reported that *E. suis* and *E. polecki* could cause severe enteritis in pigs (Matsubayashi et al. 2015b). Based on research that *E. suis* induces hemorrhage colitis until it invades the lamina propria (Matsubayashi et al. 2014). In traditional pig farming in Indonesia, most farmers pay less attention to hygiene aspects where pig manure is scattered in the yard, as well as the location of the cage adjacent to the residence, which can be the source of disease transmission to animals and humans, especially *E. histolytica*, which is a pathogenic species (Matsubayashi et al. 2015a). Several species of *Entamoeba* that have zoonotic potential are *E. polecki*, *E. histolytica,* and *E. coli* (Matsubayashi et al. 2015b; Ji et al. 2019; Wardhana et al. 2020; Pinilla et al. 2021). On the other hand, *E. polecki* has been classified into 4 subtypes from ST1 to ST4. According to Matsubayashi et al. (2015a, 2015b, 2016) *E. polecki* ST1 and *E. polecki* ST3 are associated with proliferative enteritis with co-infection by the bacterium *Lawsonia intracellularis* (Li) in pigs. Meanwhile, *E. polecki* ST2 and ST4 were detected in humans and non-human primates (Tuda et al. 2016).

Studies on identifying *Entamoeba* spp. in Indonesia are mostly done by microscopic examination that finds the stage of cysts or trophozoites in feces (Widisuputri et al. 2020), while molecular identification of parasites is still limited. Microscopic examination through morphological identification couldn't identify the parasite at the species level. This condition could cause biased diagnosis because morphological observations cannot distinguish among *Entamoeba* species. Therefore, molecular techniques with species-specific primers are the mainstay for species detection because these techniques have high specificity and sensitivity levels (Hirashima et al. 2017). Several microscopic studies on gastrointestinal parasites often found in Bali, and Papua pig farms are *Entamoeba* sp., *Balantidium* sp., *Eimeria* sp., *Blastocystis* sp., *Ascaris* sp., *Trichuris* sp., *Strongyloides* sp. and *Oesophagostomum* sp. (Widayati et al. 2020, Widisuputri et al. 2020).

The sampling location in this study was pig farms from several villages in Kupang city, which is located in East Nusa Tenggara Province, Indonesia. The area is prospectively for developing pigs for breeding and fattening. There are still traditional cage models (ground floor located near the owner's residence), but most are semi-traditional (the cage floor is made of cement, there are partial walls, and the roof is made of tile). Based on livestock and animal health statistics data (2018), the population of pigs in East Nusa Tenggara has spread across 22 districts/cities, with as many as 2,598,370 pigs in 2021 and 2022, which increased to 2,694,830 pigs. In Kupang, the total population of pigs is around 525,445 (Central Bureau of Statistics Nusa Tenggara Timur 2021).

This study aims to detect the diversity and prevalence of *Entamoeba* species in pigs in Kupang using molecular methods and the SSUrRNA gene as a marker and to detect other gastrointestinal parasites as coinfections. Meanwhile, research on gastrointestinal parasites, especially the molecular detection of *Entamoeba* spp. in the Kupang area of East Nusa Tenggara, has never been reported. Moreover, molecular detection research of *Entamoeba* spp. in pigs in Indonesia is rare. At the same time, several countries such as Japan, Korea, China, and Vietnam have found various species of *Entamoeba* in pigs, including *E. suis*, *E. polecki* ST1, and *E. polecki* ST3 showing clinical symptoms of severe diarrhea and body weaknesses. (Matsubayashi et al. 2014; Ji et al. 2019; Tuda et al. 2019; Pinilla et al. 2021).

MATERIALS AND METHODS

Ethical approval

This study protocol was reviewed by the Animal Care and Use Committee (ACUC) with ethical feasibility No. 1.KE.105.08.2021 and monitored by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga.

Study area

The geographical description of Kupang City, East Nusa Tenggara, Indonesia, is located between 10°12' latitude and 123°35' east longitude. Administratively, Kupang City consists of 6 sub-districts and 51 urban villages, with an area of 260,127 Km^2 or 26,012.7 Ha. East Nusa Tenggara is a province with the highest population of pigs, reaching 2,694,830 in 2020, while the largest population is in Kupang Regency, with 525,445 (Central Bureau of Statistics, East Nusa Tenggara 2021). East Nusa Tenggara is a province located in the southeast of Indonesia, which the Flores Sea borders in the north, the Indian Ocean in the south, Timor Leste in the east, and West Nusa Tenggara Province in the west. East Nusa Tenggara is an archipelagic province consisting of 1192 islands, most of which are uninhabited. The five large islands in East Nusa Tenggara are known as 'Flobamorata,' consisting of Flores, Sumba, Timor, Alor, and Lembata islands. Kupang city topographically consists of coastal areas, lowlands, and hills. The lowest area is located at an altitude of 0-50 meters above sea level, while the highest area is located in the southern part with an altitude between 100-350 meters above sea level. The city of Kupang is visually a lowland area where the government offices are located that has also been used for business activities, such as rainfed rice fields, seasonal gardens, and shrubs. In the southwest and south, some hills must be protected by reforestation which functions as a catchment area to maintain groundwater potential in Kupang City. The city of Kupang often dubbed the City of Coral, is a dry area, and the dry season between May and November often experiences a clean water crisis. Air temperature and humidity are determined by the altitude of the place above sea level and its distance from the coast. In 2012 the average minimum air temperature in Kupang City was 20°C-24°C (Central Bureau of Statistics 2021). The maximum air temperature occurs in November (34.8°C), and the minimum air temperature in August (20°C). The highest humidity is in January and March (88%), and the lowest is in August (62%), with an average annual humidity of 74.5% (Central Bureau of Statistics 2021).

Fecal samples collection from pigs

The pig farms and fecal samples sampling method were carried out randomly. A sum of 100 samples of pig feces of various breeds (Landrace, Yorkshire, Duroc-local) were collected from eight pig farms in Kupang, East Nusa Tenggara from eight villages, namely Baureta, Nesi Paraf, Penfui, Fatukoa, Binilaka, Camplong, Kolhua, and Tofa. Fresh fecal samples were collected immediately after defecation, and sampling was conducted in the rainy season from November 2020 to January 2021. After the stool samples were collected, the pigs' ages were recorded as follows: 13 of >6 months on farm A; 3 of 3-6 months, and 5 of > 6 months on farm B; 4 of > 6 months on farm C; 10 of > 6 months on farm D; 10 of >6 months on farm E; 1 of <3 months, 28 of 3-6 months, 11 of >6 months on farm F; 3 of 3-6 months on farm G; 4 of 3-6 months, 2 of >6 months on H. In all farms, 10-15 piglets after weaning were kept in pig pens on cement floors or soil until 3 months of age. Pigs over 3 months old were kept individually in cages. The sampling data included: owner, location, clinical symptoms, stool consistency (normal/diarrhea), age, and type of pig. The feces were stored in plastic bags at 4˚C until parasites were examined. Fecal samples taken were then put in plastic pots stored in an icebox and brought to the Laboratory of Molecular Biology and Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya.

Microscopic identification of *Entamoeba* spp. and other parasites.

Stool samples were examined under a microscope after centrifugation and observed by the floating method with saturated sugar, which uses a modified floating method based on previous research (Ekawasti et al. 2019). Stool samples were weighed 5-10 g, diluted in distilled water, and filtered with gauze. Then it was centrifuged at 1500 rpm for 10 minutes. Next, a sugar solution with a specific gravity of 1.2 was added to the sediment, and the sample was centrifuged again. Parasites floating on the surface of the sugar solution were recovered using a Pasteur pipette and washed with distilled water. Then, the purified parasites were resuspended in 1 mL PBS and stored at 4°C. A total of 15 µL of the parasitic solution was placed on a slide. The entire smear was examined with a light microscope to identify the type of parasite and calculate the degree of parasitic infection found. Parasitic morphology includes the shape, characteristics, and diameter (length and width) of worm eggs, as well as cysts and oocysts of protozoa. Positive samples for *Entamoeba* cysts were subjected to molecular identification, while other parasites were identified based on morphology (Chihi et al. 2019; Chrismanto et al. 2022).

Identification of *Entamoeba* spp. carried out on 500 µL of purified parasite aliquots. After centrifugation, 0.5-0.7 mL DNAzol® (Molecular Research Center, OH, USA) was added to the sediment, and samples were subjected to 3 freeze-thaw cycles to lyse the cyst wall; The samples were then processed according to the DNAzol manufacturer's instructions. Polymerase Chain Reaction (PCR) reactions were performed with the following primer pairs (Table 1) targeting the SSUrRNA gene for species and subtype identification. That is, 764-RD3 and 764-765 were used for the nested PCR reaction resulting in an approximately 320-bp fragment specific to *E. suis* (Clark et al. 2006). In addition, primer Epolec F6-Epolec R6 was used to generate a diagnostic approximately 430-bp fragment of *E. polecki* ST1-4 (Matsubayashi et al. 2015b). Furthermore, *E. polecki* 1 - *E. polecki* 2 was used to amplify an approximately 200 bp fragment indicating *E. polecki* ST1 (Ji et al. 2019). In addition, the genus-specific primer was used to amplify the 18S SSU rDNA for the screening of *E. coli*, yielding an amplicon of approximately 160 bp (Debenham et al. 2017). The PCR products were separated by electrophoresis using 1.5% agarose gel (Nacalai Tesque, Kyoto, Japan), stained with Gel Stain (GreenStar™ Nucleic Acid Staining Solution I, Bioner, Daejeon, Korea), and visualized using a UV transilluminator.

Table 1. Specific primers used to detect *Entamoeba* species

Species	The sequence of primers (5′-3′)	Amplicon size (bp)
E. polecki ST 1	Epolecki 1 TCG ATA TTT ATA TTG ATT CAAATG Epolecki 2 CCT TTC TCC TTT TTT TAT ATT AG	210
E. polecki ST3	F1 GTC TAT TCG ATC AAT TCAATT A R2: TAT ATT AGT CTT TTT AAAAAC TAT A	190
E. polecki	Epolec F6 AAA TTA CCC ACT TTT AAT TTA GAG AGG Epolec R6 TTT ATC CAA AAT CGA TCA TGA ATT TT	430
E. coli	F-GAATGTCAAAGCTAATACTTGACG R-GATTTCTACAATTCTCTTGGCATA	160
E. suis	F-ATC AAA TCA ATT AGG CAT AAC TA R- AAT TAA AAC CTT ACG GCT TTA AA	320

RESULTS AND DISCUSSION

The results of microscopic examination of pig feces from Kupang, East Nusa Tenggara of 100 fecal samples from 8 pig farming areas (Baureta, Nesi Paraf, Penfui, Fatukoa, Binilaka, Camplong, Tofa, and Kolhua), showed positive *Entamoeba* spp. of 98% (98/100), with a detailed examination in Table 2. A morphological examination was conducted based on the size and shape of the cyst (Lastuti et al. 2022). The degree of infection was measured by the number of *Entamoeba* cysts per gram of feces, around 10^2-10^3. Based on PCR examination using species-specific primers, detected *E. suis* was 98% (98/100) positively, which were distributed in Baureta village 13%, Nesi Paraf 8%, Penfui 4%, Fatukoa 16%, Binilaka 10%, Camplong 38%, Kolhua 3%, and Tofa 6%. The research results also found *E. polecki* at 44% (44/100) and distributed to the region of Nesi Paraf 8%, Penfui 4%, Fatukoa 10%, Binilaka 10%, Camplong 3%, Kolhua 3%, and Tofa 6%. This study also found the prevalence of *E. polecki* ST3 was 10% in Binilaka, and *E. coli* was 30%. Finding *E. polecki* ST3 and *E. coli* show that pigs in several areas in Kupang are infected with potentially zoonotic species. In addition to finding various species of *Entamoeba* by PCR amplification, based on microscopic examination, other gastrointestinal parasites were also found as coinfections, namely: *Eimeria* sp. (34%), *Cystoisospora suis* (30%), *Strongyloides* sp. (34%), *Oesophagostomum* sp. (51%), *Metastrongylus* sp. (18%), and *Trichuris suis* (8%). The results of the molecular detection of *E. suis* samples from Kupang showed a high prevalence, although the stools looked normal, and the pigs did not show any clinical symptoms. The high prevalence of *E. suis* is almost the same as the survey of *E. suis* prevalence in Tangerang, East Java, at 81.1%, as reported by Wardhana et al. (2020). In Kupang, however, detected *E. polecki* at (46%), as well as *E. polecki* ST3 (10%), were found in Binilaka and potentially zoonotic species (Wardhana et al. 2020), while the detection of *E. polecki* ST1 was not found. The PCR conducted with 1.5% gel electrophoresis of *E. coli* and *E. polecki* from samples of Kupang is shown in Figures 1 and 2. The results of the examination of *Entamoeba* spp. and coinfection is shown in Table. 2.

According to Stensvold et al. (2018), *E. polecki* is a digestive tract protozoan parasite infecting pigs, monkeys, primates, and birds which has the potential to be zoonotic. *E. polecki* has 4 subtypes (ST1-4), and the most frequently reported zoonoses are ST1 and ST3, while ST2 and ST4 are specific subtypes in humans and non-human primates (NHP). The study on pig farms in Tangerang, West Java, detected 17.3% of *E. polecki* ST3 (Wardhana et al. 2020). Similarly, *E. polecki* ST3 has been found in pigs in the Honshu Islands, Japan (Komatsu et al. 2019). The results of the exploration of pig samples from Kupang also found *E. coli* (30%) that occurred in Baureta (7%), Nesi Paraf (8%), Penfui (4%), Fatukoa (5%), and Binilaka (6%). A survey of zoonotic potential in pigs in Bucaramanga, Colombia, detected 33.7% of *E. coli* (Pinilla et al. 2021). The study conducted by Dong et al. (2017) has detected the prevalence of *E. coli* (62%) in non-human primates and potentially zoonotic. It is necessary to notice the potential for transmission to humans from animals as reservoirs. Based on the findings of swine *E. coli* and *E. polecki* ST3 in Kupang, it needed to understand the transmission of *Entamoeba*, a potentially zoonotic species. Based on sample data, positive pigs do not show symptoms of diarrhea because pigs have acquired immunity when exposed to the initial infection (innate immunity). According to Ji et al. (2019), *E. polecki* infection in animals often found in the sampling areas shows asymptomatic symptoms. If the infection is acute or in large quantities, it will cause disturbances in the digestive tract and diarrhea. Pathogenesis is getting worse due to coinfection with other pathogens, such as *Lawsonia intracellularis* (Li), which increases the disease's severity. It was previously supposed that most of the *Entamoeba* spp. non-pathogenic to swine; however, *E. suis* and *E. polecki* have been found to cause severe enteritis. *Entamoeba suis* is supposed to induce hemorrhagic colitis by invading the lamina propria (Matsubayashi et al. 2019). Moreover, *E. polecki* ST1 and ST3 are associated with proliferative enteritis caused by *Lawsonia intracellularis* (Matsubayashi et al. 2015a, 2015b, 2016).

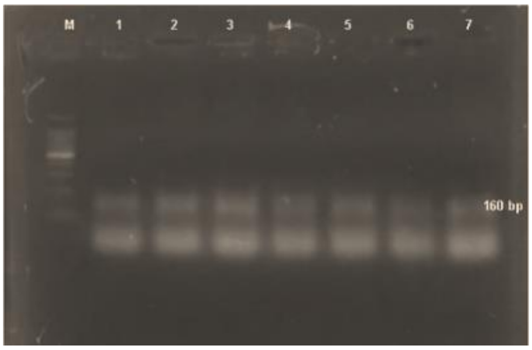

Figure 1. PCR product of *Escherichia coli* band 160bp in pigs (M: Marker, 1-7: Samples)

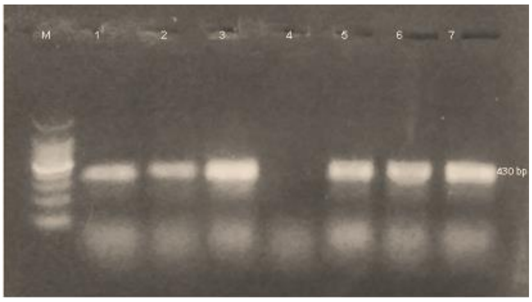

Figure 2. PCR product of *E. polecki* band 430bp in pigs (M: Marker, 1-7: Samples)

Table 2. Summary of examined samples and results for *Entamoeba* spp. and gastrointestinal parasites

Location	Age (months)	No. of animals	Microscope positive (%)	PCR analysis					Co-infection
				E. suis	*E. polecki ST 1*	*E. polecki ST 3*	*E. polecki*	*E. coli*	
Baureta (13)	< 3	-	-	-	-	-	-	-	
	3 - 6	-	-	-	-	-	-	-	
	>6	13	13	13	-	-	-	-	*Eimeria* sp., *Cystoisospora suis*, *Strongyloides* sp., *Oesophagustomum* sp.
Nesi Paraf (8)	< 3	-	-	-	-	-	-		
	3 - 6	3	3	3	-	-	3	8	*Eimeria* sp., *Cystoisospora suis*, *Strongyloides* sp., *Ascaris suum*, *Metastrongylus* sp.
	>6	5	5	5	-	-	5	-	*Eimeria* sp., *Cystoisospora suis*, *Strongyloides* sp.
Penfui (4)	< 3	-	-	-	-	-	-	-	
	3 - 6	-	-	-	-	-	-	-	
	>6	4	4	4	-	-	4	4	*Eimeria* sp., *Cystoisospora suis*, *Stronyloides* sp.
Fatukoa (16)	< 3	-	-	-	-	-	-		
	3 - 6	6	6	6	-	-	6	5	*Eimeria* sp., *Cystoisospora suis*, *Stronyloides* sp., *Oesophagostomum* sp., *Metastrongylus* sp.
	>6	10	10	10	-	-	4	-	*Eimeria* sp., *Cystoisospora suis*, *Stronyloides* sp., *Oesophagostomum* sp.
Binilaka (10)	< 3	-							
	3 -6	-							
	>6	10	10	10	-	10	10	6	*Eimeria* sp., *Cystoisospora suis*, *Stronyloides* sp., *Oesophagostomum* sp.
Camplong (40)	< 3	1	1	1	-	-	-		
	3 - 6	28	26	26	-	-	3	-	*Eimeria* sp., *Cystoisospora suis*, *Ascaris suum*, *Strongyloides* sp., *Trichuris suis*, *Metastrongylus* sp.
	>6	11	11	11	-	-	-	-	*Eimeria* sp., *Cystoisospora suis*, *Ascaris suum*, *Strongyloides* sp., *Trichuris suis*
Kolhua (3)	< 3	-	-	-	-	-	-	-	
	3 -6	3	3	3	-	-	3	-	*Eimeria* sp., *Cystoisospora suis*, *Trichuris suis*
	>6 b	-	-	-	-	-	-		
Tofa (6)	< 3	-	-	-	-	-	-		
	3 - 6	4	4	4	-	-	4	-	*Eimeria* sp., *Cystoisospora suis*, *Strongyloides* sp., *Metastrongylus* sp, *Oesophagostomum* sp.
	>6	2	2	2	-	-	2	-	*Eimeria* sp., *Cystoisospora suis*, *Stronyloides* sp., *Oesophagostomum* sp.
Total		100	98 (98%)	98 (98%)	-	10 (10%)	44 (44%)	30 (33%)	

In addition, the presence of mixed infections between *E. suis* and *E. polecki* in Kupang (44%) was quite high, as was the result of a survey by Wardhana et al. (2020) showed mixed infection from 2 to 3 species or subtypes of *Entamoeba*, confirmed 29.3% of positive samples. The survey results reported from pig farms in China detected *E. polecki* ST1 (38.2% and 45.2%) and *E. polecki* ST3 in 10.0% and 34.1%, respectively (Li et al. 2018). Moreover, Ji et al. (2019) reported a prevalence survey and molecular identification of three *Entamoeba* species in pigs in Southeast China infected by *E. suis* and *E. polecki* ST1, and *E. polecki* ST3. The overall prevalence of *Entamoeba* spp. in pigs by 55.4%, with the highest infection rate of *E. polecki* ST1 (45.2%) followed by *E. polecki* ST3 (34.1%) and *E. suis* (13.0%). Mixed infections were also reported between *E. polecki* ST1 and ST3 (25.1%), *E. polecki* ST1 and *E. suis* (3.7%), *E. polecki* ST3 and *E. suis* (0.3%), *E. polecki* ST1, ST3 and *E. suis* (4%), while piglets still suckling or growing in a predisposition to infection with *Entamoeba* spp. These differences may reflect environmental variations; for example, cysts from *Entamoeba* spp. can survive for as long as 2 weeks under humid conditions (Wardhana et al. 2020), or differences in farm management that entamoeba cysts are more easily transmitted in cages that contain about 10-15 individuals. According to Smith (2004), that cases of *E. polecki* were repeatedly found in pig dung, but no human cases were reported until 1949. Researchers believe many cases occur in humans but are not identified or misdiagnosed with other species. Furthermore, the infection often does not show clinical symptoms. Transmission of *E. polecki* through the fecal-oral route is by feces containing infective cysts that contaminate the environment, then transmit to humans who eat without washing their hands with soap after direct contact with feces or a polluted environment. Moreover, the cyst stage can last from a few days to a few weeks in the surrounding. The dense population and excessive and poor or dirty cage management support the existence of *E. polecki* protozoa (Smith 2004).

Potential risk factors for the prevalence of *Entamoeba* spp. are hygiene, socioeconomic, exposure to human and animal waste, and household wastewater (Ben-Ayed et al. 2017; Atabati et al. 2020). Desowits (1986) reported cases of amoebiasis in children caused by *E. polecki* (19%) in Papua New Guinea and estimated that pigs were the source of transmission to humans because of the close relationship between humans and pigs, and supported by poor sanitation. Research reports show that pigs play a large role in farming in China; due to the development of pig farming and high population density in China, there is a risk of zoonotic *Entamoeba* spp. unavoidable (Ji et al. 2019). It has been reported that the Nhue river water in Vietnam, which is contaminated with human and animal waste, is used intensively for agriculture; this leads to the potential of transmitting *E. histolytica* (Pham-Duc et al. 2011). Research results have reported risk factors associated with the prevalence of *E. histolytica* in three ethnic groups in Malaysia, showing a high prevalence in people with the habit of eating with their hands, consuming raw vegetables, and having frequent contact with animals (Shahrul et al. 2012). Furthermore, the study reported by Li et al. (2018) that prevalence differences can be caused by sample size, detection procedure, geographic variation, host age, sex, health status, and time of specimen collection. The infection rate by *E. histolytica* differs between countries and can be influenced by socioeconomic and sanitary conditions and population density. According to Hansen et al. (2015), that parasitic diseases caused by *E. histolytica* are highly endemic in all socio-economically poor communities. The use of wastewater and human and animal waste in agriculture and aquaculture is commonly used in China, South and Southeast Asia, and various regions of Africa, especially where water scarcity will become more severe. The main water sources for irrigation in Vietnam are freshwater, wastewater and groundwater. The use of household and human and animal waste in agriculture and cultivation has a long tradition in Vietnam. Studies in Vietnam have shown that a lack of sanitation facilities and inadequate use of fresh manure or animal manure as fertilizer in agriculture increases the risk of parasitic infection (Do et al. 2007). A study in Hanoi, North Vietnam, on the epidemiology and etiology of diarrheal diseases in adults involved in wastewater agriculture and aquaculture has shown that *Escherichia coli* and *E. histolytica* are common pathogens causing diarrhea (Do et al. 2007).

Another gastrointestinal parasite as coinfections detected microscopically is *Eimeria* sp. identified size 22.04x12.40 µm with sporulation time from 1 day to 5 days into infective oocysts in the form of oval-shaped with a smooth colorless cell membrane has 4 sporocysts, and each sporocyst consists of 2 sporozoites. In addition, some potentially zoonotic protozoa transmitted from boars to humans include *Balantidium coli*, *Entamoeba polecki*, *Blastocystis* sp., *Giardia* sp., *Cryptosporidium* sp., and *Toxoplasma gondii* (Yaghoobi et al. 2016). The *Balantidium* sp. identified in this study measures 142.83x105.73 µm, oval in shape and ciliated. These findings indicate that pigs in Kupang isolates were infected with zoonotic protozoa such as *E. polecki* ST3, *E. coli*, and *Balantidium coli*.

On the other hand, research by Yu et al. (2020) recently found an oval trophozoite measuring 50x80 µm. Based on the life cycle of *B. coli* at the trophozoite stage, it develops in the caecum and colon. It actively moves because it has cilia, penetrating the mucosa and damaging the intestinal wall, causing inflammation. While the cyst in *Balantidium* sp. has a thick wall that can survive outside the host body, it is an infective stage easily transmitted to other animals and humans (Yaghoobi et al. 2016). The study conducted by Byun et al. (2021), from 188 fecal samples by microscopic examination and PCR, showed the prevalence of *B. coli* in pigs in Korea was detected in 79 (42.9%) and 174 (94.6%). This study was also developed with PCR-restriction fragment length polymorphism (PCR-RLFP), identified 62 (33.7%) for variant A and 160 (87.0%) for variant B, as well as 48 (26.1%) coinfected samples with both variants. Based on sequence and phylogenetic analysis showed high genetic diversity of *B. coli* in pigs in Korea. Furthermore, *Cystoisospora suis* was detected in pigs in

Kupang, with the morphology of oocysts being round, measuring 19.01x17.05 µm, and there were 2 sporocysts inside the oocysts which were successfully sporulated on day 5. The research results from Joachim et al. (2018) detected using autofluorescence that the oocyst wall of *Isospora suis* emitted a sporulated blue color and measured 18x20 µm. Coccidiosis in piglets is caused by *Cystoisospora suis*, with symptoms of diarrhea, and affects suckling piglets in the first weeks after birth. The short excretory period and the high-fat content in the feces often hampered oocysts' detection in animal feces (Joachim et al. 2018). Another parasite found was *Ascaris suum* (16%) which was identified in this study, measuring 62.26x48.01 µm, in the form of a short oval in shape, brownish yellow, and jagged surface. While the research found by Taylor et al. (2016) showed egg size of *A. suum* ranged from 50-75 x 40-50µm. Baie et al. (2022) conducted a gastrointestinal parasite study from 960 samples of pigs feces by sedimentation and flotation methods, which showed samples infected with *A. suum* (63.13%) on fatteners and 34 0.06% on sows.

In conclusion, the species diversity of *Entamoeba* spp. in pigs in Kupang was found through molecular examination with partial SSUrRNA gene and species-specific primers detected *E. suis* (98%), *E. polecki* (44%), *E. polecki* ST3 (10%), and *E. coli* (30%), as well as other gastrointestinal parasites, were found as coinfections, namely: *Eimeria* sp., *Cystoisospora suis*, *Strongyloides* sp., *Oesophagostomum* sp., *Metastrongylus* sp., *Ascaris suum*, and *Trichuris suis*. With the discovery of *E. polecki* ST3 and *E. coli*, which are potentially zoonotic protozoa, as well as other parasitic coinfections. Further research is needed to understand the transmission of *Entamoeba*, a potentially zoonotic species, particularly in areas near pig farms in Kupang.

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