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PROTOZOOLOGY - ORIGINAL PAPER

Occurrence and genetic identifications of porcine *Entamoeba*, *E. suis* and *E. polecki*, at Tangerang in West Java, Indonesia

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Abstract

Entamoeba suis and *E. polecki* subtype (ST) 1 and ST3 recently have been inferred to be virulent in pigs. However, because relevant molecular epidemiological surveys have been limited, the prevalences of these species remain unknown and their pathogenicities are still controversial. We surveyed 196 fecal samples of pigs (118 of adults, 78 of piglets) at Tangerang in West Java, Indonesia, in 2017, employing PCR using porcine *Entamoeba*-specific primers. *E. suis* was the more frequently detected species, observed in 81.1% of samples, while *E. polecki* ST1 and ST3 were detected in 18.4% and 17.3% of samples, respectively; mixed infections (harboring 2–3 species or subtypes of *Entamoeba*) were confirmed in 29.3% of positive samples. Statistically significant differences in the positive rates were not seen between adult pigs and piglets, except for those of *E. polecki* ST3. The prevalences of *Eimeria* spp. and/or *Cystoisospora suis* (79.1%), strongyles (55.6%), and *Strongyloides* spp. (6.1%) were also observed morphologically in the samples. Further chronological or seasonal investigations of pigs and humans in these high-prevalence areas are needed to assess the virulence of the *Entamoeba* parasites, including the effects on pig productivity, and to evaluate the zoonotic impacts of these organisms.

Keywords *Entamoeba polecki* · *Entamoeba suis* · Gastrointestinal parasites · Indonesia · Subtype

Introduction

Parasites of the genus *Entamoeba* are known to infect members of every vertebrate class (Neal 1966; Stensvold et al. 2011). Most species of the genus typically exist in two morphological forms, trophozoites or cysts. Trophozoites serve as an active and proliferative stage in the hosts. On the other hand, cysts, a spore-like stage that can survive in the environment after being shed in feces, serve as sources of oral infection of new hosts. Classically, species within the genus have been classified based on derived hosts, morphologies (including sizes of the parasites), non-cyst or cyst formation, and the number of nuclei present in the mature cyst (one, four, or eight) (Levine 1973). More recently, however, molecular analyses have been used to distinguish species and genotypes, given how difficult it can be to distinguish among species (especially genotypes) due to their morphological similarities.

Among the genus, only two species, *E. histolytica* (and *E. moshkovskii* in some reports) in humans and *E. invadens* in

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reptiles possess invasive characteristics and cause fatal diseases; other species of the genus are considered non-pathogenic (Clark 1995; Fotedar et al. 2007; Shimokawa et al. 2012; Kyany'a et al. 2019). However, two porcine *Entamoeba* spp., *E. suis* and *E. polecki*, recently have been implicated in causing severe lesions associated with enteritis. Hemorrhagic colitis resulting from *E. suis* invasion of the lamina propria (Matsubayashi et al. 2014) and proliferative enteritis induced by *E. polecki* (associated with lethal lesions when combined with *Lawsonia intracellularis* or *Salmonella enterica* serovar Typhimurium) have been reported in Japan (Matsubayashi et al. 2015a, 2015b, 2016; Hirashima et al. 2017; Ito et al. 2020). Although *E. suis* predominantly infects pigs, *E. polecki* has been detected in multiple hosts in addition to pigs. Four genetic subtypes (ST1–4) of *E. polecki* have been defined based on the sequences of the small-subunit ribosomal RNA (SSU rRNA) genes (Stensvold et al. 2011). ST1 is found in pigs and humans; ST3 is detected in pigs, humans, and birds; and ST2 and ST4 infect humans and primates (Stensvold et al. 2018). Additionally, the susceptibility of pigs to *E. histolytica* has been demonstrated only by experimental infection of a miniature pig experimental model (He et al. 2012).

Additional studies for the detection and surveillance of porcine *Entamoeba* spp. have been conducted in several countries other than Japan. In Spain, coinfection by *E. polecki* (unknown subtype) and *Brachyspira hyodysenteriae* was reported to cause severe necrotizing lesions in pig (Cuvertoret-Sanz et al. 2019). Surveillances of *E. polecki* ST1 and ST3, in addition to *E. suis*, were performed using more than 500 fecal samples in China (Li et al. 2018; Ji et al. 2019), and parasites were detected in collections of fecal specimens (ranging in size from a few to around 10 samples each) obtained in some countries, e.g., Indonesia, Sweden, the UK, and Germany (Tuda et al. 2016; Stensvold et al. 2018; Wylezich et al. 2019). However, the pathogenicity or prevalence of *Entamoeba* infections in pig cannot be elucidated given the limited amount of data provided in those papers. Here, we examined pig feces samples collected in Indonesia, a country in which the prevalence of *Entamoeba* spp. remains unknown. Specifically, we sought to clarify the frequency of infection with *Entamoeba* spp. and assess the zoonotic potential of these isolates.

Materials and methods

Collections of fecal samples

A total of 196 porcine fecal samples were collected from 118 adults and 78 piglets. These specimens were collected at Tangerang in West Java, Indonesia, in February 2017. Tangerang is one of the cities in Banten Province, which is located around 30 km from Jakarta (the capital city of Indonesia) (Fig. 1). Geographically, this city is 0–25 m above sea level, the annual temperature is 23–34 °C with a relative humidity of 80.0%, and the average annual rainfall is around 154.9 mm. The examined pigs were located on 29 farms in 7 villages in 5 districts. Aside from exhibiting, in some instances, slowed growth, these animals showed no apparent clinical symptoms at the time the feces were collected (Table 1 and Fig. 1) and the animals were not treated with any anthelmintic. Most of the farms in the present study traditionally reared about a few to 10 adult pigs including mother pigs and piglets (a total of approximately less than 100 pigs). The pig pens were constructed using bricks or bamboo fences and were located in the backyard of or next to the houses of those rearing the animals. The floors were mainly concrete, but pens on a few farms had dirt floors. Fresh feces, which were collected within a few hours after being shed, were placed in individual plastic bags and stored at 4 °C until used in the laboratory as described below.

DNA purification and molecular identification of *Entamoeba* spp.

For DNA extractions of *Entamoeba* spp., individual fecal aliquots (200 mg each) were mixed with 0.5–0.7 mL of DNAzol® (Molecular Research Center, OH, USA). Samples then were processed according to the DNAzol manufacturer's instructions, except that after being diluted with this reagent, samples were subjected to 3 freeze-thaw cycles to disrupt the cysts. Using the resulting extracted DNA as templates, PCR reactions were performed with the following primer pairs targeting the SSU rRNA gene for species and subtype identifications: 764-RD3 and 764-765 were employed for nested PCR reactions that yielded an approximately 320-bp fragment specific to *E. suis* (Clark et al. 2006); Epolec F6-Epolec R6 were used to generate an approximately 430-bp fragment diagnostic of *E. polecki* (Matsubayashi et al. 2015b); Epolecki 1-Epolecki 2 were used to amplify an approximately 200-bp fragment indicative of *E. polecki* ST1 (Verweij et al. 2001); EpST3 F1-R2 were employed to generate a 190-bp fragment specific for *E. polecki* ST3 (Hirashima et al. 2017). Additionally, primers EhL-EhR and EdL-EdR (from multiplex primer sets targeting *Entamoeba* SSU rRNA genes) (Evangelopoulos et al. 2000) were used to screen for *E. histolytica* in a few extracted samples from each farm. Amplicons were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. PCR products of reactions using primers 764-765 (for a total of 67 extracted samples, using 1–3 samples per positive farm (28 positives of 29 examined farms) because of the large number of *E. suis*-positive samples) and Epolec F6-Epolec R6 for sequences of *E. polecki* subtypes (ST1–4) (all of the 56 *E. polecki* ST1 and ST3 specific PCR-positive samples (22

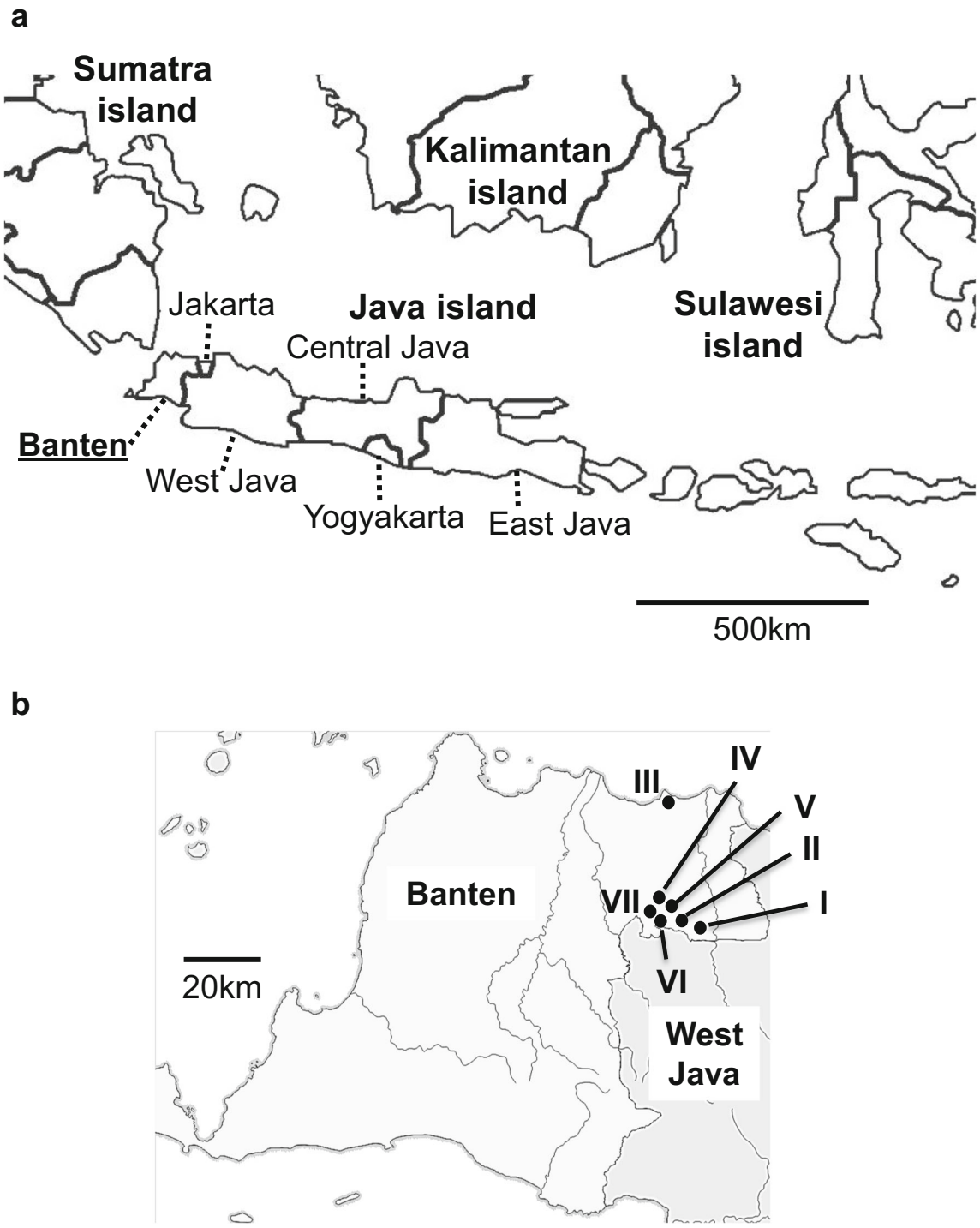

Fig. 1 Maps showing the main islands in Indonesia (Java, Sumatra, Kalimantan, and Sulawesi) (**a**) and the locations of villages on Banten that were surveyed (**b**). The villages from which samples were collected included Dandang (I) in Cisauk; Cirarab (II) in Legok; Marga Mulya (III) in Mauk; Ciakar (IV), Mekar Jaya (V), and Ranca Iyuh (VI) in Panongan; and Margasari (VII) in Tigaraksa. The numbers in Fig. 1b correspond to the designation used in Table 1

ST1 positives, 20 ST3 positives, and 14 both ST1 and ST3 positives)) were purified using a Gel/PCR™ DNA Isolation System (Viogene, New Taipei, Taiwan). The purified fragments were subjected to two-directional DNA sequencing, and the sequences were aligned and subjected to homology searches as described previously (Matsubayashi et al. 2014). A phylogenetic tree was constructed using the neighbor-joining algorithm with evolutionary distances computed using the Tamura-Nei model and 1000 bootstrap replicates. The resulting tree was

Table 1 Summary of examined areas and results for *Entamoeba* spp. and gastrointestinal parasites*[1]

No.	Sub District	Village	No. of farms	No. of examined animals Adult / piglets	No. of negative*[2] Adult / piglets	PCR analysis			Flotation methods			
						E. suis	*E. polecki* ST1	*E. polecki* ST3	*Eimeria* spp. /*Cystoisospora suis*	Strongyles	*Strongyloides*	Others
I	Cisauk	Dandang	3	NA 19	NA 0	NA 18	NA 4	NA 9	NA 18	NA 5	NA 1	NA 4 (*Ascaris suum*)
II	Legok	Cirarab	2	NA 24	NA 3	NA 17	NA 5	NA 5	NA 18	NA -	NA 0	NA 0
III	Mauk	Marga Mulya	13	63 2	2 0	48 2	3 0	4 0	45 2	55 2	4 2	0
IV	Panongan	Ciakar	2	NA 10	NA 0	NA 8	NA 3	NA 3	NA 8	NA 3	NA 0	NA 0
V		Mekar Jaya	1	11 8	1 1	9 7	0 3	0 2	7 2	6 1	0 0	0 1 (*Trichuris suis*)
VI		Ranca Iyuh	2	4 13	0 0	4 12	1 1	1 1	4 10	2 9	0 0	0 0
VII	Tigaraksa	Margasari	6	40 2	0 0	34 0	16 0	9 0	39 2	26 0	4 1	5 (*Ascaris suum*), 1 (*Trichuris suis*) 0
Subtotal			29	118 78	3 4	95 (80.5) 64 (82.1)	20 (16.9) 16 (20.5)	14 (11.9) 20 (25.6)*[3]	95 (80.5) 60 (76.9)	89 (83.1) 20 (25.6)*[3]	8 (6.8) 4 (5.1)	
Total (%)				196	7 (3.6)	159 (81.1)	36 (18.4)	34 (17.3)	155 (79.1)	109 (55.6)	12 (6.1)	

*[1] Upper and lower values within a given cell indicate numbers of adult pigs and piglets, respectively

*[2] Animals negative for all tested parasites

*[3] Statistically significant differences between adult pigs and piglets ($P < 0.05$)

NA: not available

drawn using the MEGA software package (version 5; Tamura et al. 2011).

Screening for gastrointestinal parasites

Separate aliquots of each fecal sample were subjected to a flotation method using Whitlock universal chambers. The entire field was inspected using light microscopy, and the numbers of detected *Eimeria* and/or *Cystoisospora* oocysts and then nematode eggs were quantified (Gordon and Whitlock 1939; Ekawasti et al. 2020).

Statistical analyses

The differences in the prevalence of the parasites between adult pigs and piglets were evaluated using Fisher's exact test ($P < 0.05$).

Results

We examined a total of 196 pig fecal samples using PCR reactions targeting porcine *Entamoeba* spp. The fecal specimens also were inspected for selected gastrointestinal parasites using standard flotation methods. Parasites were detected at all of the examined farms; only 3.6% of the examined pigs were negative for all tested parasites. Among the organisms for which we screened, *E. suis* was detected most frequently (in 81.1% of the animals); *E. polecki* ST1 and ST3 were detected in 18.4% and 17.3% (respectively) of the pigs (Table 1). In total, *Entamoeba* spp. were detected in 28 of 29 examined farms. The prevalences of *Entamoeba* spp. were similar in adult pigs compared with those in piglets except for those of *E. polecki* ST3; mixed infections (consisting of 2–3 species/subtypes of *Entamoeba* per animal) also were detected (Table 2). PCR analysis demonstrated that none of the examined samples harbored *E. histolytica*. Based on the flotation analysis, coccidian parasites, *Eimeria* spp., and/or *Cystoisospora suis* were detected at high frequency (in 79.1% of examined pigs); strongyles (55.6%), *Strongyloides* spp. (6.1%), and a few other parasites (e.g., *Ascaris suum* and *Trichuris suis*) also were found in the animals. Although the parasite species could not be defined completely based solely on morphologies of the detected oocysts and eggs (e.g., *Eimeria* spp. and *C. suis*, strongyles, and *Strongyloides* spp.), the numbers per gram of feces were counted successfully. The mean numbers of the oocysts or eggs per gram of feces for adults and piglets (respectively) were 3147 (range 77,600 to 40) and 3232 (range 25,280 to 80) for *Eimeria* spp. and/or *C. suis*; 716 (range 9800 to 40) and 4522 (range 68,400 to 40) for strongyles; and 755 (range 3760 to 40) and 1390 (range 3120 to 320) for *Strongyloides* spp. These results suggested that piglets shed more parasites in feces than did adult pigs. There were statically significant differences between adult pigs and piglets ($P < 0.05$) in the prevalence of *E. polecki* ST3 and strongyles.

Sequence analyses of amplicons obtained using the 764-765 or Epolec F6-Epolec R6 primer pairs successfully yielded nucleotide sequences for all of the extracted 67 *E. suis*–positive samples, and 14 of 36 *E. polecki* ST1–positive samples and 16 of 34 *E. polecki* ST3–positive samples, respectively. All of the sequences were identical among the *E. suis*–positive isolates and among the *E. polecki* ST3–positive isolates, and all of these partial sequences were identical to the respective sequences previously reported for *E. suis* and *E. polecki* ST3 (e.g., Accession Nos. LC230019 and FR686385, respectively). The *E. polecki* ST1 isolates yielded two distinct sequences differing by three nucleotides. One class of ST1 sequence (designated ST1-1) was identical to previously published data (e.g., Accession No. MK801460), and the other (designated ST1-2) was identical to that of an Indonesian isolate (Accession No. LC082305) (Tuda et al. 2016). A phylogenetic tree was constructed using the SSU rRNA gene sequences derived in the present work, in combination with published sequences for the respective genes from *Entamoeba* spp. (Fig. 2). The sequences of *E. polecki* ST1 and ST3 isolates each formed a clade with sequences of the respective subtypes, but given their small size (less than 300 bp), the partial sequences of *E. suis* could not be incorporated into this phylogenetic tree.

Table 2 Summary of the number of detected *Entamoeba* spp. and subtypes

Species and subtypes*	No. of samples (%)
E. suis	111 (66.5)
E. suis + *E. polecki* ST1	18 (10.8)
E. suis + *E. polecki* ST3	17 (10.2)
E. suis + *E. polecki* ST1 + ST3	13 (7.8)
E. polecki ST1	4 (2.4)
E. polecki ST3	3 (1.8)
E. polecki ST1 + ST3	1 (0.6)
Total	167

*ST, subtype

Discussion

Using PCR with species- and subtype-specific primers, we surveyed villages in a limited area of Indonesia for porcine *Entamoeba* sp. infections. The prevalence of *E. suis* (81.1%) was elevated compared with values (13.0% and 0.8%) reported for China; on the other hand, *E. polecki* ST1 (18.4%) and

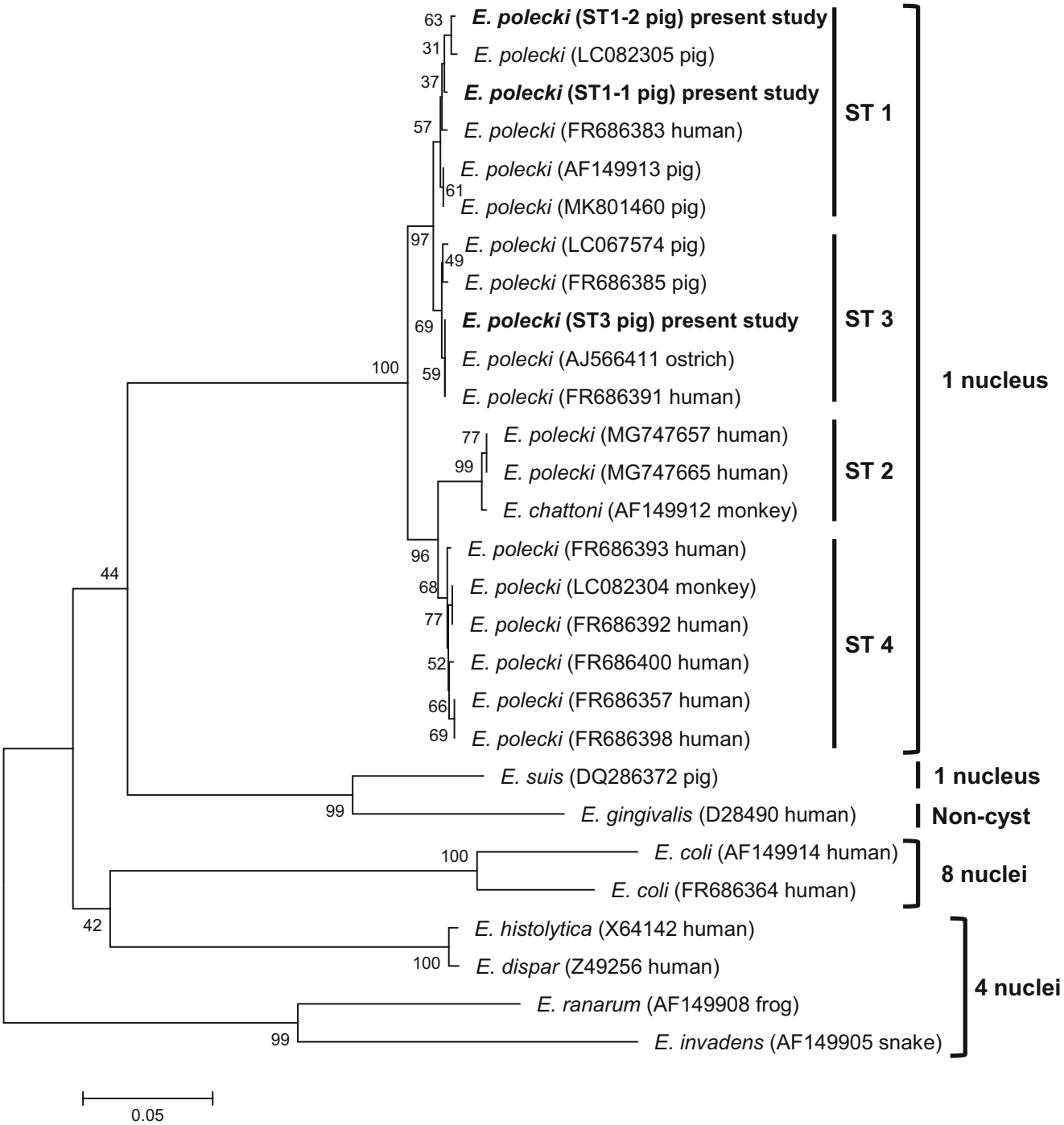

Fig. 2 Phylogram of *Entamoeba polecki* subtypes (ST) 1–4, related parasites and other *Entamoeba* spp. inferred by the neighbor-joining method using partial SSU rRNA gene sequences. Accession numbers and derived hosts are shown in parentheses. Scale bar represents substitutions per nucleotide, and bootstrap values are indicated (> 1000)

ST3 (17.3%) were detected at prevalences that were lower than those reported for China (38.2% and 45.2% for ST1, 10.0% and 34.1% for ST3) (Li et al. 2018; Ji et al. 2019). These differences may reflect environmental variations (e.g., cysts may survive for longer periods under moister conditions) or distinctions in farm management (e.g., the cysts may be more easily transmitted in facilities housing larger populations of pigs). Thus, further investigations in other countries and in other areas of Indonesia will be needed to clarify these differences in prevalence.

Coccidian parasites were detected at a high prevalence (79.1%) that was similar in magnitude to that of *E. suis*.

Notably, the transmission mechanisms for *Entamoeba* spp. and for *Eimeria* and *Cystoisospora* spp., namely via fecal-oral routes for the robust cysts and oocysts, are almost identical (Petri and Singh 1999; Schuster and Visvesvara 2004; Joachim et al. 2018). Floors in most of the examined farms were concrete, and thus, concrete flooring would be expected to reduce the ingestion of intermediate hosts (worms) by the pigs, resulting in an observation consistent with the low prevalence observed for *Strongyloides* spp. (6.1%). The farmers cleaned such surfaces regularly (if not daily) to remove the feces (data not shown). However, based on the frequency at which we confirmed infection by *Entamoeba* spp. by PCR and/or coccidian parasites by the floatation methods, although we could not examine the presence of the cysts by staining with iodine, the pigs presumably still come into contact with cysts and oocysts before removal of feces, or cysts and oocysts (which show environmental resistance) persisted despite floor washing.

Sequencing revealed the presence of two sequence types of *E. polecki* ST1 (ST1-1 and ST1-2), along with *E. polecki* ST3; each of these sequences or subtypes formed their clusters in phylogenetic analyses. The sequences for the *E. polecki* ST1-2 isolates identified in the present study were identical to those previously obtained for the organism infecting a pig on a different island in Indonesia (Tuda et al. 2016). These observations indicate that *E. polecki* ST1-1, ST1-2, and ST3 may be widespread among pigs of Indonesia. However, further surveys of additional pigs and areas will be needed to evaluate the geographic prevalence of various *Entamoeba* species, subtypes, and sequence types. The present study did not assess the pathogenicity of these porcine isolates. Given that *E. suis* and two subtypes of *E. polecki* are inferred to have pathogenic effects in pigs, it will be necessary to chronologically or seasonally investigate pigs to assess parasite virulence, including effects on pig productivity and on the health of humans living in high-prevalence areas. These wider surveys will be critical to evaluating the zoonotic transmission and clinical impact of *Entamoeba* in Indonesia.

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Authors' contributions All authors contributed equally in writing the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval All experiments were carried out by examinations of fecal samples without using live vertebrate animals. Thus, ethical approval for animal experimentation was not necessary. Field hygienic surveys of pig farms were conducted by veterinarians who were employed as civil servants by prefectural governments; all of these personnel belonged to the Livestock Hygiene Service Centre. All examinations performed in this study took place with the permission of the farm owners and were conducted as part of government affairs. No animals were sacrificed for the purposes of this study. No human participants were involved in this study.

Consent for publication All authors consent to the publication of the manuscript in *Parasitology Research*.

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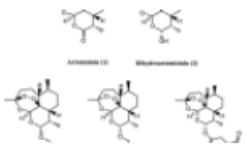

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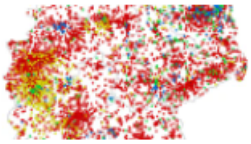

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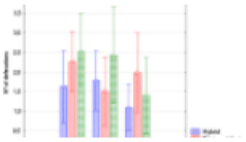

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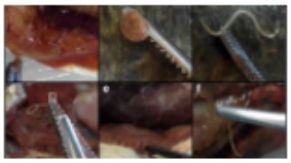

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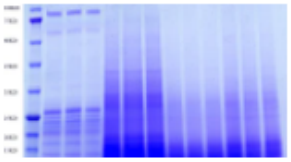

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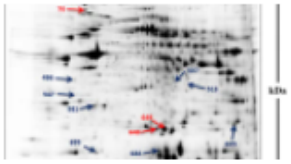

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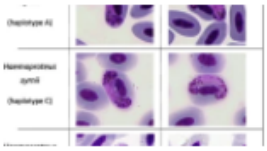

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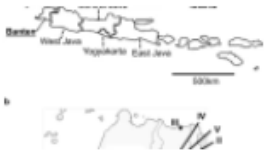

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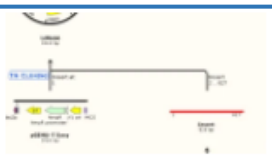

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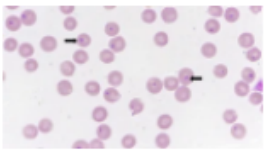

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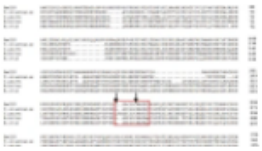

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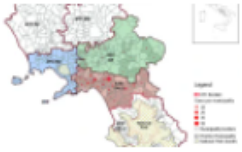

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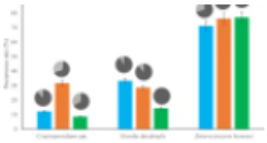

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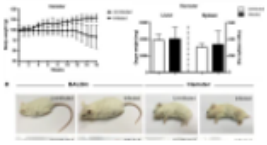

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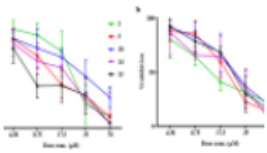

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