

Case of Entamoebiasis in Pigs Raised with a Free Range Systems in Bali, Indonesia

(KASUS ENTAMOEBIASIS PADA BABI YANG DIPELIHARA DENGAN CARA DIUMBAR DI BALI, INDONESIA)

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ABSTRACT

This research aims were to measure the prevalence of *Entamoeba* in pigs in Bali and to identify the zoonotic potential species of *Entamoeba*. A total of 183 pig stool samples from Bali have been examined. The method being used in this study were combination between coproscopy and molecular techniques. Concentration sedimentation with Sodium Acetic Formaldehyde (SAF) as a solution was used in the coproscopy method, while the Polimerase Chain Reaction method was used to amplify DNA of *Entamoeba*. Extracted sample's DNA examined by using primers that specifically for *Entamoeba*: Entam 1 (F) (5'-GTT GAT CCT GCC AGT ATT ATA TG-3') and Entam 2 (R) (5'-CAC TAT TGG AGC TGG AAT TAC-3), and to identify the zoonotic potential species of *Entamoeba*, samples that produce 550 bp in first amplification continued by primers Epolecki1 (F) (5'-TCG ATA TTT ATA TTG ATT CAA ATG-3') and Epolecki2 (R) (5'-CCT TTC TCC TTT TTT TAT ATT AG-3'). The results showed that 76.6% of samples were positive in coproscopical examination, but 84.7 % produced 550 bp bands on PCR amplification by using general primers. All positive samples on the first PCR continued to second PCR used specific primers for *E. poleckii* as a potential zoonotic disease and all of the samples showed negative results. This data demonstrated that the prevalence of *Entamoeba* in a traditional pig scavenging systems in Bali was 84.7% but no specific infection infection caused by *E. polecki* was found.

Keywords: *Entamoeba*, pigs, Bali.

ABSTRAK

Penelitian ini bertujuan untuk mengetahui prevalensi *Entamoeba* pada babi di Bali serta mengetahui spesies yang potensial sebagai agen zoonosis. Sebanyak 183 sampel feses babi yang diambil dari seluruh Bali telah diperiksa. Metode yang dipergunakan pada penelitian ini adalah kombinasi metode koproskopi dan molekuler. Pemeriksaan koproskopi menggunakan metode konsentrasi sedimentasi dengan *Sodium Acetic Formaldehyde* (SAF) sebagai pelarut sampel, sedangkan untuk uji molekuler dipergunakan *Polimerase Chain Reactions* (PCR) untuk amplifikasi DNA *Entamoeba*. DNA sampel yang telah diekstrak, diuji menggunakan primer yang mampu mendeteksi *Entamoeba* secara umum yaitu Entam 1 (F) (5'-GTT GAT CCT GCC AGT ATT ATA TG-3') dan Entam 2 (R) (5'-CAC TAT TGG AGC TGG AAT TAC-3). Untuk menentukan spesies yang bersifat zoonosis, sampel yang menghasilkan pita dengan bobot molekul 550 bp dilanjutkan dengan PCR menggunakan primer spesifik untuk *E. polecki* yaitu Epolecki1 (F) (5'-TCG ATA TTT ATA TTG ATT CAA ATG-3') and Epolecki2 (R) (5'-CCT TTC TCC TTT TTT TAT ATT AG-3'). Hasil penelitian menunjukkan sebanyak 76,6% sampel positif terinfeksi oleh *Entamoeba* pada pemeriksaan koproskopi, sedangkan pada uji PCR, sebanyak 84,7% sampel memproduksi 550 bp band pada amplifikasi menggunakan primer umum. Selanjutnya pada amplifikasi menggunakan primer spesifik *E. polecki*, seluruh sampel menunjukkan hasil negatif. Hasil ini mengindikasikan bahwa prevalensi *Entamoeba* pada babi yang dipelihara dengan sistim pemeliharaan tradisional di Bali adalah sebesar 84,7% namun tidak ditemukan adanya infeksi oleh *E. polecki* yang bersifat zoonosis.

Kata-kata kunci: *Entamoeba*, babi, Bali

INTRODUCTION

More than 90% of Balinese are Hindu, they need pigs in almost every religious process. Pigs are one of the most important livestock in Bali, as they have traditional, religious and economic values. The population and demand of pigs in Bali are increasing every year. As many as 924.297 pigs were reported in 2011 in Bali (Sumantra, 2011), this amount was believed to rise in the next years. Pigs have an important role for the public, such as: as a source of protein, alternative income and saving, employment and produced fertilizer. Pigs have many advantages over other livestock, for examples: the growth rate is relatively faster, easier to breed and to find sources for feed and their carcass value is quite high (Nugroho and Whendrato, 1990).

Unfortunately, people in Bali are still applying a traditional pig scavenging system. They live with their pigs without any limit and sometimes stay in the same building. Pigs defecate anywhere and pollute the environment. This close association between pigs and humans enables cross infection with a range of parasites to occur (Krauss, 2003). Common zoonotic parasite diseases transmitted by pigs are *Taenia solium*, *Trichinellosis*, *Toxoplasmosis*, *Ascariasis* and *Entamoebiasis*, all of which contribute deleteriously to human health (Velmurugan *et al.*, 2009; Nejsun *et al.*, 2012; Stensvold *et al.*, 2010; Yanagida *et al.*, 2012; Papatsiros *et al.*, 2012).

Duc *et al.*, 2011 reported that worldwide human cases of *Entamoebiasis* were around 500 million people per year and 40.000-10.000 people died, most of the cases occurred in children (1-5 years old) (Germani *et al.*, 1994; Nelson, 2000). Diarrhea is the most common clinical feature in infected people in Indonesia. The incidence tends to increase in the rainy season. The morbidity of diarrheal diseases in Indonesia was 19.5%, and it was the highest number cases in South East Asia countries (Sunoto, 1990). The data showed the incidence of diarrhea in Indonesia is very high, nearly 60 million cases per year where 60 to 80% of diarrhea suffered by children under the age of five years (Depkes RI, 2005). Sutanto *et al.* (2008) reported 8 to 18% of diarrheal cases caused by *Entamoeba*.

Common diagnostic method used to identify the species of *Entamoeba* is coproscopy to distinguish the number of nuclei in the cell. However, it could not highly confirm the result of the samples (Verweij *et al.*, 2003). Molecular

methods have been successfully developed to identify the species of *Entamoeba* (Vianna *et al.*, 2009). The *Entamoeba* species that has a zoonotic potential is *E. polecki* (Desowitz and Barnish, 1986; Mohamadi and Petri, 2006), but no data on the prevalence of *E. polecki* in pigs in Bali was reported. Therefore, measuring the prevalence and identifying of the zoonotic potential species of *Entamoeba* are necessary.

RESEARCH METHODS

A total of 183 pig stool samples collected from all regencies in Bali, each sample preserved into two different solutions; sodium acetic formaldehyde for coproscopy methods and potassium dichromate for molecular methods. All samples in SAF solutions examined by coproscopy method (Burrows, 1959; Marti and Escher, 1990) and molecular techniques (Verweij *et al.*, 2001; Sukprasert *et al.*, 2008).

DNA extraction followed instruction of Stoll Extraction Kit by Qiagen® 2007. The PCR mixture consisted of 200 µM (each) deoxy-nucleoside triphosphates, 1x PCR buffer, 1.5 mM MgCl₂, 0.5 U of *Taq* polymerase, and 12.5 pmol of forward and reverse primers in a total 25 µL reaction mixture. PCR process used two different primers; first PCR used the primers for general *Entamoeba*: Entam 1 (F) (5'-GTT GAT CCT GCC AGT ATT ATA TG-3') and Entam 2 (R) (5'-CAC TAT TGG AGC TGG AAT TAC-3), Amplification was performed with predenaturation condition at 95°C for three min, followed by 35 cycles with the following reaction conditions: denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and polymerization at 72°C for 30 sec. At the end of the polymerase was added at 72°C for two min (Sukprasert *et al.*, 2008). Positive samples at first PCR running produced 550 bp band, than the samples were continued to second PCR by using specific primers for *E. polecki*: Epolecki1 (F) (5'-TCG ATA TTT ATA TTG ATT CAA ATG-3') and Epolecki2 (R) (5'-CCT TTC TCC TTT TTT TAT ATT AG-3'). Amplification was performed with predenaturation condition at 94°C for five min, followed by 35 cycles with the following reaction conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and polymerization at 72°C for 30 sec. At the end of the polymerase was added at 72°C for two minutes. Five µL of all PCR products in 2 µL loading dye continued to process electrophoresis 1.5 % of gel at 90 volt for 35 min (Verweij *et al.*, 2001).

RESULTS AND DISCUSSION

Among 183 stool samples analyzed by using optical microscopy, 119 (76.8%) samples indicated having *Entamoeba* forms. The *Entamoeba*'s cyst size ranges from 9.5 to 15.5 μm (mean $12.85 \pm 1.72 \mu\text{m}$ based on 100 cysts) (Fig 1). The simplest morphological feature to be used in *Entamoeba* species identification was the number of nuclei per cyst. Normally, the species produces cysts with one, four or eight nuclei and a few do not encyst. A previous phylogenetic study of *Entamoeba* species (Silberman *et al.*, 1999) suggested that this morphological feature reflected the phylogenetic relationships among organisms, with the species producing cysts with different numbers of nuclei forming distinct clades. However morphological

descriptions are often incomplete and figures unclear, leaving doubts as to the validity of the names and making comparisons between species described by different individuals virtually impossible in some cases. *Entamoeba* species are not very diverse at the morphological level, with cyst size, number of cyst nuclei and appearance of the chromatoidal bars (crystal line arrays of ribosomes) being the main identification criteria (Clark *et al.*, 2006).

Therefore, because of many forms of *Entamoeba* which would be difficult to be identified and need more specific and sensitive methods for the diagnosis (Singh, 1997). While DNA amplification used general *Entamoeba* primers resulted 155 (84.7%) samples produced 550 bp band (Fig 2). Regarding molecular analysis of *Entamoeba* isolates, discrepancy between microscopy and genetic analysis results could be due to both inhibitory problems of PCR and too low sensitivity/specificity of microscopic techniques (Levecke *et al.*, 2010).

These findings appear comparable with previous data available in the literature. The prevalence of *Entamoeba* in free-range pigs in Kenya was 87% (Kagira *et al.*, 2010). Suryawan *et al.* (2014) reported that 80% of pig farms in Papua were infected by *Entamoeba spp* with prevalence was 32.4%. But this result is higher than data that Damriyasa and Bauer (2006) reported, they found 15 from 20 pig breeding farms in Southern Hesse, Central Germany were infected by *Entamoeba*, with prevalence 52% in unweaned piglets. Low prevalence reported in Korea with 3.7% in rural areas of Chungcheongnam-do (Ismail *et al.*, 2010). The prevalence of *Entamoeba* in other animals also

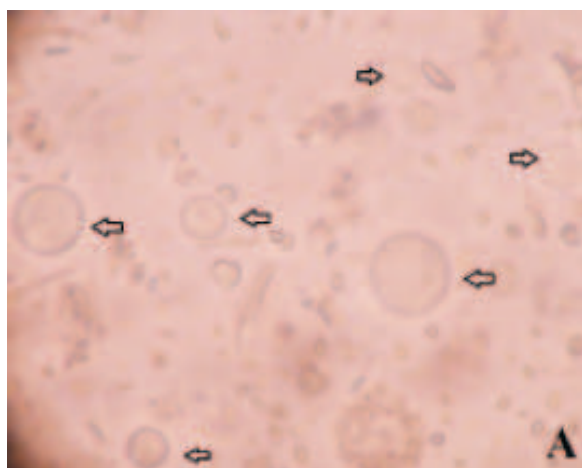


Figure 1. Entamoebal form in coproscopy method (400x zoom)

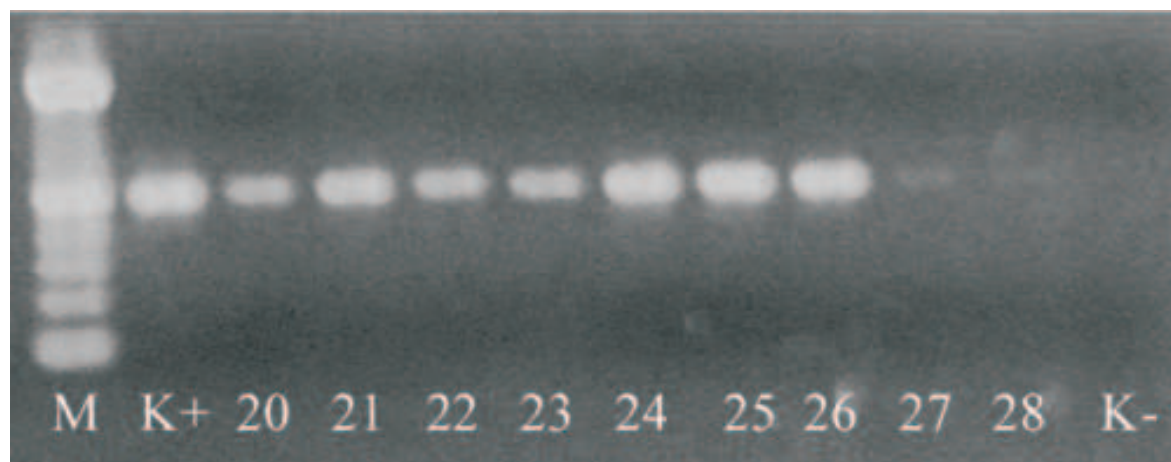


Figure 2. Amplification used general Entamoeba primers produced 550 bp band. M: DNA ladder 100 bp, K+: Positive control, 20 to 26: Positive samples, 27 to 28: Negative samples and K-: Negative control

reported; 81.2% in non-human primates in an Italian zoological garden (Berrilli *et al.*, 2011), 81.1% in United Kingdom (Regan *et al.*, 2014) and Levecke *et al.* (2007) reported the prevalence among Old World monkey was ranging from 30% to 100% and among apes ranging from 0% to 100%.

From a public health point of view, these protozoa have high zoonotic potential, being among the most common intestinal human parasites worldwide (Stauffer and Ravdin, 2003). Zoonoses issues worldwide spread from countryside to the larger community in the world that affects epidemiology. This happens due to the high rate of urbanization, land conversion and poor sanitation (Kraus, 2003). A zoonotic disease that is related to the poor sanitation and personal hygiene is *Entamoebiasis* caused by *E. polecki* (Mohamadi and Petri, 2006).

Enteric protozoa infection is one of diarrheal diseases in children. Intestinal protozoa are transmitted by the fecal-oral route and exhibit life cycle consisting of a cyst stage and a trophozoite stage. The cysts consist of a resistant wall and are excreted in the feces. The cyst wall functions to protect the organism from desiccation in the external environment. Unhygienic conditions promote transmission of most protozoa (Gascon *et al.*, 2000; Youssef *et al.*, 2000). Traditional patterns and semi-intensive pig farming system that is still used in Bali (Agustina, 2013) that can easily lead to the contamination of feed by cyst of *Entamoeba* spp. Bali is a tropical area with adequate rainfall which is a very good factor for the spread of infection due to *Entamoeba* spp. This cysts morphologically grow in the tropics and is able to survive for two days at 37°C while the trophozoite can survive at temperatures between 5 to 37°C and cysts are resistant to temperatures around freezing but not drought resistant (Jaco *et al.*, 2003). Cyst can survive for prolonged period in the external environment because of the protection by their cell wall (Markell *et al.*, 1999).

In this study, no samples were identified as *E. polecki*, this indicates the possibility amount of DNA produced is lower than the minimum limit that is able to be detected using this method (Bakir *et al.*, 2003). Conversely, Verweij *et al.* (2003) found 1 of 20 human fecal samples in Ghana. However, the results showed the high prevalence of *Entamoeba* in pigs in Bali, which may be *E. suis*, this agent may have implication for the health status of the pigs (Levine, 1990;

Mohamadi and Petri, 2006; Berrilli *et al.*, 2011).

A number of other potential zoonotic species of *Entamoeba* have been reported in humans included *E. polecki* which can be transmitted from pig feces (Mohamadi and Petri, 2006). Human cases of infection with the uninucleated cyst-producing *Entamoeba* species referred to as *E. polecki* are considered to be rare (Chacin-Bonilla *et al.*, 1992), except in Papua New Guinea, where prevalence rates as high as 30% are reported (Desowitz and Barnish, 1986), but instead (Pakandl, 1994; Mohammadi *et al.*, 2004) reported the prevalence of infection may reach up to 25% in wild and domestic pigs all around the world.

E. polecki from a pig in England was obtained several years ago, before the description of *E. struthionis* from farmed ostriches in Spain (Ponce Gordo *et al.*, 2004). Remarkably, there is only one base difference between this pig *Entamoeba* sequence and that deposited as *E. struthionis* in the over 1000 bases sequenced for the former. Clark *et al.* (2006) suggested that *E. polecki* is not restricted to pigs and humans but can infect birds also, and that the name *E. struthionis* is a synonym of *E. polecki*. This is further supported by the observation that the *E. struthionis* sequence is not basal to the available *E. polecki* and *E. chattoni* sequences in the phylogenetic tree but rather is specifically related to that of *E. polecki* in most analyses.

CONCLUSION

The prevalence of *Entamoebiasis* in a free range pigs in Bali Indonesia was 84.7%, but no identified infection was caused by *E. polecki*.

SUGGESTIONS

Based on the results of this research, it is necessary to use more sensitive and specific methods to identify the species of *Entamoeba* sp.

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