DETECTION OF INTESTINAL PARASITES AND *Balantidium coli* IN FARMED PIGS IN KELANTAN VIA MICROSCOPY AND MOLECULAR TECHNIQUES

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DETECTION OF INTESTINAL PARASITES AND *Balantidium coli* IN FARMED PIGS IN KELANTAN VIA MICROSCOPY AND MOLECULAR TECHNIQUES

by

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CERTIFICATE

This is to certify that the dissertation entitled "Detection of intestinal parasites and *Balantidium coli* via microscopy and molecular techniques in farmed pigs in Kelantan" is the bona fide record of research work done by Miss Foong Tong Ling during the period from September 2013 to May 2014 under my supervision.

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FOONG TONG LING

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

B. coli	*Balantidium coli*
bp	Base pair
DNA	Deoxyribonucleic acid
x *g*	Relative centrifugal force
g	gram
IC	Internal control
kg	Kilogram
µl	Microlitre
µm	Micrometer
µM	Micromolar
mg	Miligram
mL	Mililitre
mm	Millimetre
PCR	Polymerase Chain Reaction
%	Percent
s	Second
sp.	Species
18S rRNA	18S ribosomal RNA
TAE buffer	Tris-acetate-EDTA buffer

PENGESANAN PARASITI USUS DAN *Balantidium coli* DALAM KHINZIR DI KELANTAN MENGGUNAKAN KAEDAH MIKROSKOP DAN MOLEKULAR

ABSTRAK

Khinzir didapati di semua kawasan zon tropika dan zon subtropikal dunia ini. Haiwan ini ialah perumah kepada banyak jenis parasit, termasuk *Balantidium coli* yang bersifat zoonosis. Parasit-parasit usus memudaratkan kadar tumbesaran dan status kesihatan khinzir, lalu mengurangkan pendapatan penternak khinzir. Selain itu, parasit zoonosis seperti *B. coli* boleh menjangkiti manusia seperti penternak dan penduduk yang tinggal berhampiran dengan ladang khinzir. Kajian awal ini bertujuan untuk mengenal pasti helminth dan/atau protozoa usus pada 33 ekor khinzir yang dikenal pasti secara rawak di Kelantan. Selain itu, asai tindak balas untaian polimeras (PCR) dupleks juga dibangunkan untuk mengesan *B. coli* dalam sampel tinja khinzir. Kaedah smir basah dan pemekatan formalin eter diguna untuk mengesan parasit usus dalam spesimen tinja. Pengesanan DNA *B. coli* dikendalikan dengan mengamplifikasi gen 18S rRNA *B. coli.* Gen *Plasmodium falciparum* diguna sebagai kawalan dalaman untuk memastikan keputusan PCR yang negatif adalah betul-betul negatif dan bukan disebabkan oleh perencat PCR. Hasil keputusan menunjukkan 78.8% khinzir adalah positif dengan satu atau lebih jenis parasit usus. Untuk smir basah, telur *Ascaris* sp., ova-ala *Trichuris* sp., sista-ala *Balantidium* dan parasit anu didapati dalam 13 (39.4%), 3 (9.1%), 3 (9.1%) and 14 (42.4%) specimen tinja masing-masing. Kaedah pemekatan formalin eter menunjukkan 15 (45.5%) spesimen adalah positif dengan *Ascaris* sp., 12 (36.4%) dengan ova-ala *Trichuris* sp., dan 17 (51.5%) dengan parasit anu. Selain itu, ketiga-tiga spesimen yang mengandungi

sista-ala *Balantidium* didapati negatif selepas diuji dengan PCR dupleks. Kesimpulannya, kajian ini memunjukkan banyak khinzir di Kelantan dijangkiti dengan ascariasis dan trichuriasis, tetapi bebas daripada jangkitan *B. coli*.

DETECTION OF INTESTINAL PARASITES AND *Balantidium coli* IN FARMED PIGS IN KELANTAN VIA MICROSCOPY AND MOLECULAR TECHNIQUES

ABSTRACT

Pigs are found throughout the tropical and temperate regions of the world. These animals are hosts to numerous intestinal parasites, which include the zoonotic *Balantidium coli*. Intestinal parasites cause detrimental effect to the growth rate and health status of pigs, which ultimately decrease the income of pig farmers. In addition, zoonotic parasite such as *B. coli* harbors by pigs may infect farmers and people who stay near pig farms. Hence, this preliminary study aimed to identify intestinal helminths and/or protozoa in pigs reared in Kelantan. An in-house duplex polymerase chain reaction (PCR) assay was developed to detect *B. coli* in the stool specimens. A total of 33 pig faecal specimens were randomly collected from farmed pigs in Kelantan. Direct wet mount and formalin ether concentration methods were used to examine the stool specimens for presence of intestinal parasites. DNA detection of *B. coli* was performed using an in-house duplex PCR assay that amplified 18S rRNA gene of *B. coli*. *Plasmodium falciparum* gene was incorporated as the internal control to rule out the presence of PCR inhibitors in false negative DNA specimens. Results showed that 78.8% of the pigs were positive with one or more parasites. By direct wet mount, *Ascaris* sp., *Trichuris* ova-like sp., *Balantidium*-like cyst and other unknown parasites were found in 13 (39.4%), 3 (9.1%), 3 (9.1%) and 14 (42.4%) specimens, respectively. Formalin ether concentration method showed that 15 (45.5%) specimens were positive for *Ascaris* sp., 12 (36.4%) for *Trichuris* ova-like sp., and 17 (51.5%) contained unknown

parasites. Interestingly, all the 3 positive stool samples for *Balantidium*-like cysts were negative for *B. coli* when screened with the in-house duplex PCR assay. In conclusion, this study showed that many of the local Kelantan pigs were infected with *ascariasis* and *trichuriasis* but none were infected with the zoonotic *B. coli.*

CHAPTER 1

INTRODUCTION

1.1 Literature Background

Zoonoses are diseases that can be transmitted from animals to humans. Parasitic zoonoses are transmitted to humans either by ingesting environmentally transmissive stages such as spores, cyst, oocyst, ova and larval or by eating raw meat containing infective tissue stages (Slifko *et al.*, 2000). Parasitic infections have been transmitted to humans since ages. The number of protozoan and helminthic parasites that caused human infections has increased over time. There are about 300 helminthic and 70 protozoan pathogens which cause diseases in humans. However, only about 90 species of the parasites are common (Cox, 2002). Protozoal infections cause significant morbidity and mortality throughout the world. The incessant increase of immunocompromised patients worldwide during the last few decades have led to the emergence of new protozoal pathogens (Parija and Giri, 2012). Besides that, other factors including the increase in international travel, deforestation, increase in urban dwellings and increase trade of meat, milk and other product of animal origins also contributed to parasitic diseases (Solaymani-Mohammadi and Petri, 2006; Parija and Giri, 2012)

The common zoonotic gastrointestinal parasites are *Ascaris* spp., *Trichuris* spp., *Oesophagostomum* spp., coccidia, *Giardia* spp., *Entamoeba* spp., *Toxoplasma* spp., *Sarcocystis* spp., *Balantidium* spp. and *Cryptosporidium* spp. which are usually communicable to human and animal, and vice-versa (Weng *et al.*, 2005; Bauri *et al.*, 2012). Domesticated animals are common hosts to zoonotic parasitic diseases, hence pose a threat to human health.

Balantidium coli is the largest protozoan and the only ciliate parasite that infects human. The infection is common in pigs, and the prevalence rate of 60-90% have been reported in animals in a single herd (Bauri *et al.*, 2012). Pigs, also called hogs or swine, are ungulates and have been domesticated as a source of food and leather since ancient times. Due to the impacts on agriculture, biodiversity, and human health, pigs have been labeled as "triple threat pests" (Hampton *et al.*, 2004; Hampton *et al.*, 2006). They are animals found throughout the tropical and temperate regions of the world. Over the past decade, swine industry has witnessed an unprecedented increase in production and consumption as more pork is consumed than other types of meats (Pam *et al.*, 2013). Pork consumption varies widely among countries and regions with per capita intake in 1998 ranging from 2kg/year in many African countries to 60 kg/year in Germany (Pam *et al.*, 2013).

In human, the protozoan parasite can be found in individuals who were frequently in contact with swine and those exposed to poor and low hygienic environment (Bauri *et al.*, 2012). Disease caused by *B. coli* in human usually affects the mucosa of the large intestine, but it can also invade the liver and the lungs, though it rarely happens. In this research, the focus is on intestinal parasites, especially *B. coli* that can be found in pigs.

1.1.1 Historical Perspective of *Balantidium*

Malmsten was the first to recognize the organism in two humans with dysentery in the year 1857. Malmsten identified the organism as *Paramecium* species and named it as *Paramecium coli* (Schuster and Avila, 2008). The first record of *B. coli* as a parasite of pigs was made by Leuckart in 1861. In 1862, Stein also studied *B. coli* from pigs and he was the first to assign them to the genus *Balantidium*. The genus

had been established by Claparede and Lachmann in 1858 with *Balantidium entozoon* from the frog as the type species (McDonald, 1922)

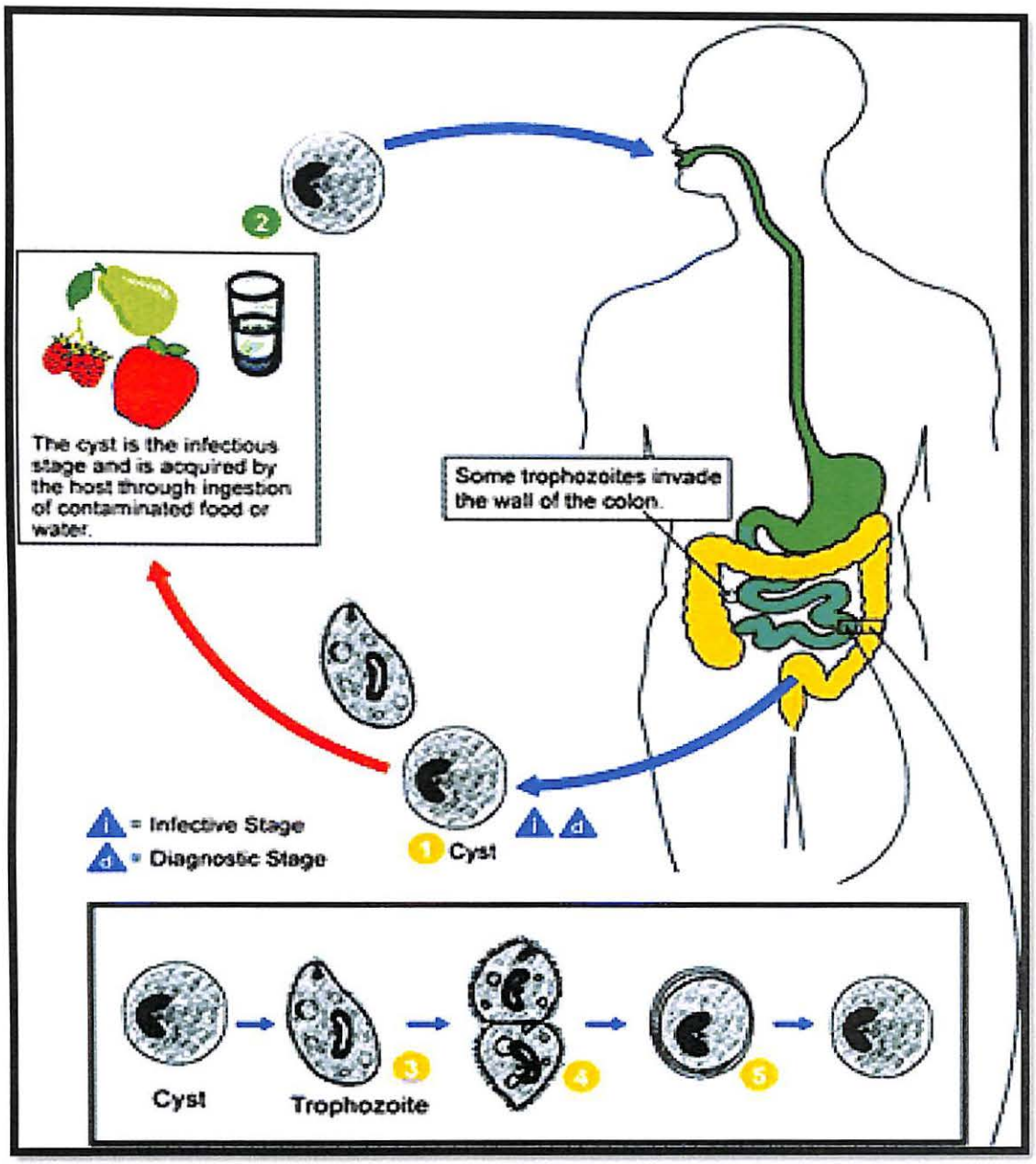
Malmsten first described balantidiosis from the disease of tropical or subtropical regions, from patients in Sweden (Schuster and Avila, 2008). This protozoan is barely visible to the unaided eye. The homogeneity of the cell contents is present with the nuclei, the contractile vacuoles, the food vacuoles, and sometimes by the presence of highly refractile bodies (McDonald, 1922). There are two forms of *B. coli*, namely trophozoite and cyst. The trophozoite is oval in shape; at the pointed anterior end is a mouth (cytosome) which leads into the cytopharynx occupying about a third of parasite length. At the more rounded posterior end is the anus, cytophage and covered with short cilia. It measures about 50 to 100 μm in length and 40 to 70 μm in width. The spherical or ellipsoid cyst is about 50 to 70 μm in diameter. It is covered with a thick and refractile cyst wall (Schuster and Avila, 2008; Wikipedia, 2014). Both forms have a large kidney-shaped nucleus (macronucleus) which is responsible for vegetative functions, and a smaller spherical nucleus (micronucleus) which is responsible for sexual reproduction (Periago, 2003).

1.1.2 Life Cycle of *Balantidium coli*

The infection begins when a host (human or animals) acquires the cyst through ingestion of contaminated food or water. The ingested cysts are capable of surviving in the acidic environment of the stomach while the basic condition of the small intestine is where excystation takes place (Liu, 2012). Motile trophozoites move with their cilia in a rotary type motion, colonize the lumen of the large intestine (cecum and colon), and feed on the intestinal flora and nutrients (Percival *et al.*, 2013).

During asexual transverse binary fission, the mother cell elongates and forms a tranverse structure through the middle. The elongated mother cell constricts in the middle and finally separates into two asymmetrical daughter individuals; an anterior and a posterior cell (Figure 1.1). The anterior cell retains the oral apparatus and develops a new excretory pore while the posterior cell generates a new mouth (Liu, 2012). Two successive fissions are followed by the formation of conjugatants at which they exchange micronuclear products of meiosis for a few moments, and then detach (Liu, 2012). Thus, new trophozoites are formed, which occur through binary fission and conjugation.

In the lumen, trophozoites may disintegrate or undergo encyctsation, which is caused by dehydration of the intestinal contents or in feces which is outside the host. Cysts are discharged with the faeces and released into the environment for onward transmission to new hosts.



(Source: URL: http://cmr.asm.org/content/21/4/626.full)

Figure 1.1 Life cycle of *Balantidum coli.* The cyst is the infectious stage and is acquired by the host through ingestion of contaminated food or water. The cyst undergoes excystation in the small intestine to form trophozoites. Trophozoites invade lumen of large intestine and undergo encycstation to form cysts, which will be excreted together along with faeces.

1.1.3 Balantidiasis

Balantidiasis is a zoonotic disease caused by *B. coli*, which is regarded as a commensal of the large intestine of pigs. The parasite has also been reported in camels, horses, cattle, rodents, nonhuman primates and human (Solaymani-Mohammadi and Petri, 2006). Despite causing no symptoms in pigs, pigs-to-pigs transmission is very common, especially in piggeries with poor hygienic conditions and poor water systems. Infected pigs may shed large amount of *B. coli* cysts in their faeces and contaminate water resources or food (Liu, 2012). Without proper water sanitation or proper cleaning food, cysts are consumed with the contaminated water or food by human. Thus, balantidiasis can be known as food and water-borne diseases (Slifko *et al.*, 2000). Humans in close contact with pigs are vulnerable to infection through fecal oral route (Bauri *et al.*, 2012).

B. coli cause no serious disease of gastrointestinal tract. It seldom causes dysenteric symptoms such as severe diarrhea and bloody stools in immunocompetent patients. However, the problems are more severe in malnutrition, alcoholism or immunocompromised patients such as patients infected with human immunodeficiency virus, HIV (Anargyrou *et al.*, 2003; Ferry *et al.*, 2004). The parasitic protozoa can be found in all age categories of pigs. Some pigs may suffer moderate to severe diarrhoea, while others show no symptoms of disease (Kennedy, 2006).

The major factors that lead to human balantidiasis include close contact between pigs and humans, lack of waste disposal system of swine and human excrement that contaminate water sources and food, changes of climatic conditions either warmth or

humidity which suits cyst survival, host factors with resistance and/or possible immunity and the etiologic agent itself and its ability to invade host tissues (Schuster and Avila, 2008; Liu, 2012).

1.1.3 (a) Epidemiology

Balantidium is the only ciliated and largest protozoan known to infect humans. It also commonly infects non-human primates, cattle, camels, horses, rats, hamsters and dogs (Swartzwelder, 1950; Schuster and Avila, 2008; Liu, 2012). However, pigs have a particularly high rate of infection (20–100%) and consequently known as main natural reservoir of this parasite (Solaymani-Mohammadi *et al.*, 2004). Human infection is common in communities that live in close proximity to pigs, such as in central and South America, Iran, Papua New Guinea and Philippines (Schuster and Avila, 2008).

B. coli infection is rare in humans, but its potential for worldwide distribution cannot be denied. This organism is considered pathogenic with low virulence. The worldwide prevalence is estimated at 0.02 to 1% (Esteban *et al.*, 1998), but varies in different geographical areas (Anargyrou *et al.*, 2003) in which regions of Latin America, Philippines, Papua New Guinea and West Iran show high prevalence of the infection (Solaymani-Mohammadi *et al.*, 2004). In New Guinea, the rate of infection among swine herders and slaughterhouse workers was 28% (Owen, 2005; Solaymani-Mohammadi and Petri, 2006), while at Altiplano of Bolivia, balantidiasis rates ranged between 6% and 29% (Esteban *et al.*, 1998). Weng *et. al* (2005) had carried out a study on the prevalence of intestinal parasites in intensive pig farms in Guangdong Province, China. From the faecal samples of 3636 pigs, 1716 pigs

(47.2%) were infected with *B. coli* (Weng *et al.*, 2005). In a study of rural areas of Chungcheongnam-do, Korea, 88 out of 136 pig samples were infected with balantidiasis (Hassan *et al.*, 2010). In another study, a high prevalence of *B. coli* infection (93%) was found in pigs in Jharkhand, India (Bauri *et al.*, 2012; Parija and Giri, 2012).

The incidence of human infection is higher where pigs share habitats with humans and faecal contamination of food and water occurs. The largest outbreak of balantidiasis occurred due to typhoon in the island of Truk in Micronesia, where many of the residents fed pigs infected with *B. coli* (Schuster and Avila, 2008). Typhoon destroyed homes and rooftop catchment systems for rainwater collection causing people without a source of clean water. They were being forced to use water from streams and wells that were contaminated with pigs and human feces and this incidence resulted balantidiasis in 110 persons in the island (Schuster and Avila, 2008). Balantidiasis is an uncommon disease in humans as it is restricted to tropical and subtropical regions due to sanitary standards, climate conditions and cultures.

1.1.3 (b) Clinical Features

Balantidiasis can induce a range of mild to severe clinical presentations, from asymptomatic, chronic, to fulminating infections (Baskerville *et al.*, 1970). Most of the infected people are asymptomatic. The infected individuals shed cysts or trophozoites in their faeces, but are free of any significant clinical symptoms. They become carriers of the disease and serve as reservoirs of infection in the community (Esteban *et al.*, 1998). For chronic infection of *B. coli*, individuals have mucoid and rarely bloody diarrhea; have alternating periods of constipation, cramping, halitosis,

nausea, foul breath, colitis and inflammation of the colon and abdominal pain (Ladas *et al.*, 1989; Liu, 2012). Besides that, balantidiasis can induce fulminating acute infection. This form of balantidiasis appears suddenly and with great intensity. Explosive diarrhea may occur as often as every 20 minutes. Dysentery may lead to severe dehydration, perforation of the colon, fever, peritonitis, pleuritis, ulcer, liver abscess, pneumonia, weight loss, shock and death especially in immunocompromised and malnourished individuals (Ferry *et al.*, 2004; Fletcher *et al.*, 2012; Liu, 2012). It has been reported to have case fatality rate of about 30% (Esteban *et al.*, 1998). Patients may die of fulminating dysentery with hemorrhage and shock resembling amoebic dysentery (Ferry *et al.*, 2004).

In the year 2003, Sharma and Harding reported a case of *B. coli* causing a thick-walled on the upper lobe cavity of the right lung in a 42-year-old organic farmer who had contact with aerosolized pig manure and traveled to the South American countries of Paraguay and Argentina (Sharma and Harding, 2003). In another study, a 59-year-old woman suffering from chronic lymphocytic leukemia was found to have pulmonary balantidiasis (Anargyrou *et al.*, 2003). Direct microscopic examination of bronchoalveolar lavage (BAL) showed that ciliated trophozoites of *B. coli* were presence. Administration of metronidazole led to rapid improvement of the condition after 24-48 hour treatment (Anargyrou *et al.*, 2003).

In Turkey, there was a 47-year-old female patient with non-Hodgkin's lymphoma who was presented with *B. coli*-associated diarrhea and abdominal pain (Yazar *et al.*, 2004). In another case, a patient was reported to experience watery, bad smelling, bloody diarrhea (10 times/ day) and abdominal pain. Moving trophozoites with cilia

were observed in stool samples and later diagnosed as *B. coli*. After treatment with 750 mg metronidazole three times daily for five days, the symptoms disappeared rapidly and the patient completely resolved (Yazar *et al.*, 2004; Liu, 2012).

1.1.3 (c) Pathogenesis

Upon ingestion by a host, *B. coli* cysts withstand acidic condition in the stomach and alkaline condition in the small intestine, and undergo excystation to become trophozoites. For invasive cases, trophozoites penetrate the epithelium from the bottom of the crypts. Balantidia produce no known toxins, but its ability to produce proteolytic enzyme, hyaluronidase which digest hyaluronic acid that is important for supporting and holding mucosal epithelial cells together in intestinal tissue. This enzyme breaks down the intestinal epithelium, resulting in ulceration of the colon wall followed by hemorrhagic lesions, perforation, secondary infection or local generalized peritonitis (Tempelis and Lysenko, 1957).

Infection of *B. coli* in pigs often presents non-specific symptoms, as such pigs are often known as asymptomatic carriers. These asymptomatic pigs serve as the major hosts for the parasite and shed vast volumes of the parasite in their faeces (Periago, 2003; Solaymani-Mohammadi and Petri, 2006). *B. coli* infection of dogs and rats is rare, and invasion of the tissues and other sites of body in these species are even less frequent. Primates may possess some natural resistance to *B. coli* infection and disease. It was reported that two monkeys treated with hydrocortisone and infected with *Balantidium* cysts developed diarrhoea, while two untreated monkeys developed only the asymptomatic infection (Yang *et al.*, 1995; Periago, 2003).

1.1.3 (d) Treatment and Prevention

B. coli is sensitive to a number of drugs, including metronidazole, tetracycline, iodoquinol, nitazoxanide, secnidazole, tinidazole, trimethoprim, sulfamethoxazole, sulfaguanidine and sulfadimidine (Liu, 2012). Currently, tetracycline, metronidazole and iodoquinol are the drugs of choice for treatment of balantidiasis in humans (Solaymani-Mohammadi and Petri, 2006). Treatment with metronidazole requires a period of 5 days (adult dosage, 750 mg three times per day; pediatric dosage, 35 to 50 mg kg of body weight^{-1} day^{-1} in three doses). Tetracycline is often prescribed for 10 days with 500 mg four times a day (for adult) and 40 mg kg^{-1} dose^{-1} in four doses (for pediatric). Iodoquinol is given for 20 days (650 mg three times daily for adult; 40 mg kg^{-1} dose^{-1} in three doses) (Yazar *et al.*, 2004; Solaymani-Mohammadi and Petri, 2006; Schuster and Avila, 2008). Because most balantidiasis cases are asymptomatic, it is important to treat asymptomatic individuals as well to avoid further transmission of the disease.

For treatment of swine balantidiasis, different therapeutic regimes including chloroquine, niridazole (ambilhar) and oxytetracycline (terramycin) have been used (Solaymani-Mohammadi and Petri, 2006). It seems that terramycin is more effective in treatment of swine balantidiasis. In one study at Zaire, at a dose rate of 15 mg/kg body weight of terramycin administered twice daily. clinical recoveries in all of the symptomatic pigs were recorded (Mwamba and Pandey, 1977).

Since balantidiasis is transmitted by a faecal-oral route, it can be preventable by improving hygiene practices, water sanitation and proper disposal of faecal excrement. Pigs as the main animal reservoir of *B. coli* cysts should be fenced and