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In vitro Development of *Cystoisospora suis* in Swine Kidney Cell Line, SK-6

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Abstract. *Cystoisospora suis (syn. Isospora suis)* is an apicomplexan parasite which causes an important piglet coccidiosis resulting in commercial losses worldwide. Successful coccidiosis control using chemoprevention and chemotherapy as the recommended approach, nonetheless, conduces the extensive use of anticoccidial drugs that may lead to the emergence of drug-resistant parasites. Consequently, it is necessary to continuously engage in drug discovery for alternative anticoccidials. To achieve the drug discovery aspect, an appropriate *in vitro* model for the endogenous development of the coccidium has to be established. Moreover, the *in vitro* model can reduce the cost and experimental animal use necessitated by *in vivo* systems. The aim of this study was to investigate the development of *C. suis* throughout its endogenous life cycle using swine kidney cell line, SK–6 for the *in vitro* system. The results showed that SK–6 cells seemed to be an excellent support system for the complete endogenous life cycle of *C. suis*. For ability to infect, sporozoites were able to penetrate into the host cells, first observed by 2 hours post inoculation. The intracellular penetrated sporozoites underwent asexual multiplications producing presumed type I meronts and merozoites. The meronts each formed 2–8 zoites by 3 days post infection (dpi) which were prominently seen on 3–5 dpi. Developing oocysts were initially observed on 5 dpi and went on sequential sexual development resulting in intracellular mature oocysts from 7–14 dpi. These became prominent from 9–12 dpi. Recycling of merozoites to form more meronts and merozoites also appeared by 10 dpi, indicated by merozoites burst from initially infected cells observed re-entering host cells. In conclusion, the presented *in vitro* system was able to support complete endogenous development of *C. suis* that was comparable to its *in vivo* life cycle. Furthermore, this provides an appropriate model for the study of a vast array of aspects relating to the coccidium, especially in applications with regard to the sexual development of the parasite.

INTRODUCTION

Apicomplexan parasites are pathogens of various important diseases in domestic pets, farm animals, and humans and are recognized as important economically in veterinary and medical fields (Lindsay *et al*., 1997; Kijlstra & Jongert, 2008; Müller & Hemphill, 2013). Diseases associated with coccidian infections are responsible for significant losses in animal production worldwide, especially in the food animal industry. *Cystoisospora suis*, renamed as such by Barta *et al.* (2005), was previously known as *Isospora suis* since 1934 (Biester & Murray, 1934). *C. suis* is considered to form the most serious coccidiosis in piglets due to its virulence resulting in dramatic commercial losses (Meyer *et al*., 1999; Mundt *et al*., 2005; Mundt *et al*., 2006). The most important recommendation for parasitic disease control of porcine coccidiosis is

chemoprevention and/or chemotherapy with integrated appropriate managements. Disinfectants, especially a combination of chemicals which are able to penetrate into the coccidian oocysts, have been used with success. Recommended prophylaxis and/or metaphylaxis against coccidiosis, and chemotherapy for piglets with such clinical manifestations, nowadays utilize anti-coccidial triazin derivatives (Mundt *et al.*, 2007). However, the extensive use of anticoccidial agents could result in selection of resistant parasites (Sangster, 2001) which may lead to a need for alternative new anticoccidial drugs. In order for discovery of new drugs, it is appropriate that an *in vitro* model for infection and development of the coccidium be established to reduce costs and use of experimental animals. Besides the drug discovery perspective, an *in vitro* culture system provides generally an excellent tool for testing other coccidian aspects, *e.g.* coccidian biology and studies of factors influential in the developmental stages of the coccidian parasite life cycle. Several cell types had been proposed for use in cell culture types to support growth and development of *C. suis*: primary porcine kidney and primary foetal bovine kidney cells (Lindsay & Blagburn, 1987), embryonic bovine trachea, Madin-Darby bovine kidney, porcine kidney, and bovine colon cell lines (Fayer *et al.*, 1984), diploid swine testicle cell line (Lindsay *et al.*, 1998), as well as, intestinal porcine epithelial cell line (Worliczek *et al.*, 2013). Initial studies showed some host cell penetration and intracellular development of the parasite, but limited to replication by asexual multiplications, while complete endogenous development was later exhibited by Lindsay *et al.* (1998) and Worliczek *et al.* (2013). It is important to state that reproducible development in cell culture, especially for sexual developmental stages to form oocysts and their subsequent sporulation, remains to be explored and requires further studies in understanding and control of the coccidian parasite and other related organisms.

The present study was conducted to investigate the intracellular development of *C. suis* endogenous life cycle in an alternative cell line, being swine kidney cell line SK–6. This cell line is well established and is widely used as a routine evaluating system for swine epithelial cell pathogens, especially for viruses.

MATERIALS AND METHODS

Parasite and sporozoite preparation

A *C. suis* field strain was isolated from diarrheic stool of piglet from a small farm located in a suburb of Khon Kaen, Thailand in 2006 and the isolated oocysts were stored and maintained at 4–8°C until use. The purified sporozoites used for the *in vitro* infection and development study were prepared from *in vivo* experimentally produced oocysts of the *C. suis* field strain, prior to the *in vitro* study. For oocyst production, colostrum deprived piglets were experimentally inoculated with 10 000 sporulated oocysts of *C. suis* (Mundt *et al.*, 2006). Faecal samples were collected daily during 5–12 days post infection (dpi) and a direct smear of each faeces was examined microscopically for oocysts. Only faeces which had more than 10 oocysts seen under 400x magnification were subjected to harvest using saturated sodium chloride floatation technique. After the floating period, the oocyst-rich portion at the top convex of the salt solution was collected into sterilized distilled water (SDW) followed by repeated washes by centrifugation at 1 000 x g for 10 minutes with SDW, at least 5 times. The oocyst-rich pellets of all positive faeces were pooled, partially disinfected by 5–6% (w/v) sodium hypochlorite, providing available chlorine, using Clorox® regular bleach (The Clorox Company, Oakland, USA) for 10 minutes in an ice-chilled bath, and subsequently washed with SDW by centrifugation as described above. Sporulation, excystation, and sporozoite preparation methods previously described for *Eimeria tenella* (Tomley, 1997) were adapted to *C. suis*. Briefly, oocysts were sporulated in 2% (w/v) potassium dichromate (BDH laboratory supplies, Poole, England) in the dark at room temperature. The sporulated oocysts were excysted by

mechanical cracking, followed by treatment with 1% (w/v) taurocholic acid and 0.25% (w/v) trypsin (all, Sigma-Aldrich Co., St. Louis, USA) at 37ºC for 1–2 hours to release sporozoites from the sporocysts. The sporozoites were then purified using a hybrid nylon wool and diethylaminoethyl (DEAE) cellulose column separation technique. Finally, the purified sporozoites were suspended in inoculation medium (as stated below) for *in vitro* infection and development study.

All applicable guidelines for the care and use of experimental animals of the Animal Ethics Committee of Khon Kaen University in accordance with the Ethics of Animal Experimentation of the National Research Council of Thailand were followed for artificial propagation of oocysts in piglets.

Infection and development of *C. suis* in Swine Kidney cell line (SK-6)

SK-6 cell line was kindly gifted by the Veterinary Research and Development Centre (Upper Northeastern Region), Khon Kaen, Thailand. It was subcultured and maintained in cell culture flasks (Nunc™, Nalge Nunc International, Roskilde, Denmark) containing Minimum Essential Medium (MEM) (Gibco®, Invitrogen Corporation, New York, USA) pH 7.4 supplemented with 5% foetal bovine serum (FBS) (Gibco®, Invitrogen Corporation, New York, USA), 200 units/ml of penicillin, and 20 mg/ml of streptomycin (all, Sigma-Aldrich Co., St. Louis, USA) as culture medium at 37ºC with 5% CO_2. After trypsinization of the flask cell monolayers, the cells were resuspended in culture medium. They were then seeded on cell culture coverslips, 13 mm in diameter, (Nunc™ Thermanox™, Nalge Nunc International, New York, USA) laid in each well of a 24 well cell culture plate (Nunclon™, Nalge Nunc International, Roskilde, Denmark) at 20 000 cells/well, and incubated at 37ºC with 5% CO_2. After incubation for 24 hours, 50 000 sporozoites suspended in inoculation medium consisting of MEM pH 7.4 supplemented with 5% FBS, 1 000 units/ml penicillin, and 100 mg/ml streptomycin were added to each well. At 24 hours pi (hpi), inoculation medium containing any excess free sporozoites in each well was discarded. The wells were washed 2–3 times with MEM pH 7.4, and then filled with fresh culture medium over the cell monolayer. The cultured coverslips were harvested daily from 2 hpi – 14 dpi and then subjected to cyto-staining with hematoxylin and eosin (H&E) stain (BDH laboratory supplies, Poole, England). After staining, coverslips were mounted on microscope glass slides and the cell monolayers subsequently examined under a light microscope. The stages of intracellular parasites were morphologically categorized conforming to Lindsay *et al.* (1983) and Lindsay & Blagburn (1987).

RESULTS

Host cells entering and asexual divisions

Sporozoites of *C. suis* were able to invade the epithelial cells, first observed at 2 hpi (Fig. 1A). The intracellular penetrated sporozoites became blunt and rounded in shape, seen by 1 dpi (Fig. 1B), and underwent asexual replications. The sporozoites developed within the cytoplasm of the host cell to become merozoites and meronts by 3 dpi. The merozoites and meronts were presumed to be type I according to their size and shape, which were prominently observed on days 3 to 5 pi (Fig. 1C). The formed meronts contained 2–8 merozoites which were approximately 2.6–3.3 x 8.5–9.2 µm in size. Recycling of merozoites to form new generations of merozoites and meronts also appeared at 10 dpi evidenced by merozoites observed re-entering the host cells. After re-entering the host cells, the merozoites became elongated and crescent-shaped in the parasitophorous vacuole (PV) (Fig. 1F) and then developed new generations of meronts and merozoites followed by further sexual developmental stages.

Sexual developments

At 5 dpi, underdeveloped oocysts were first observed in the host cells with oocysts being observable for the remainder of the investigation period (5–14 dpi) (Fig. 1D). The consequent stages of sexual development resulting in intracellular mature oocysts

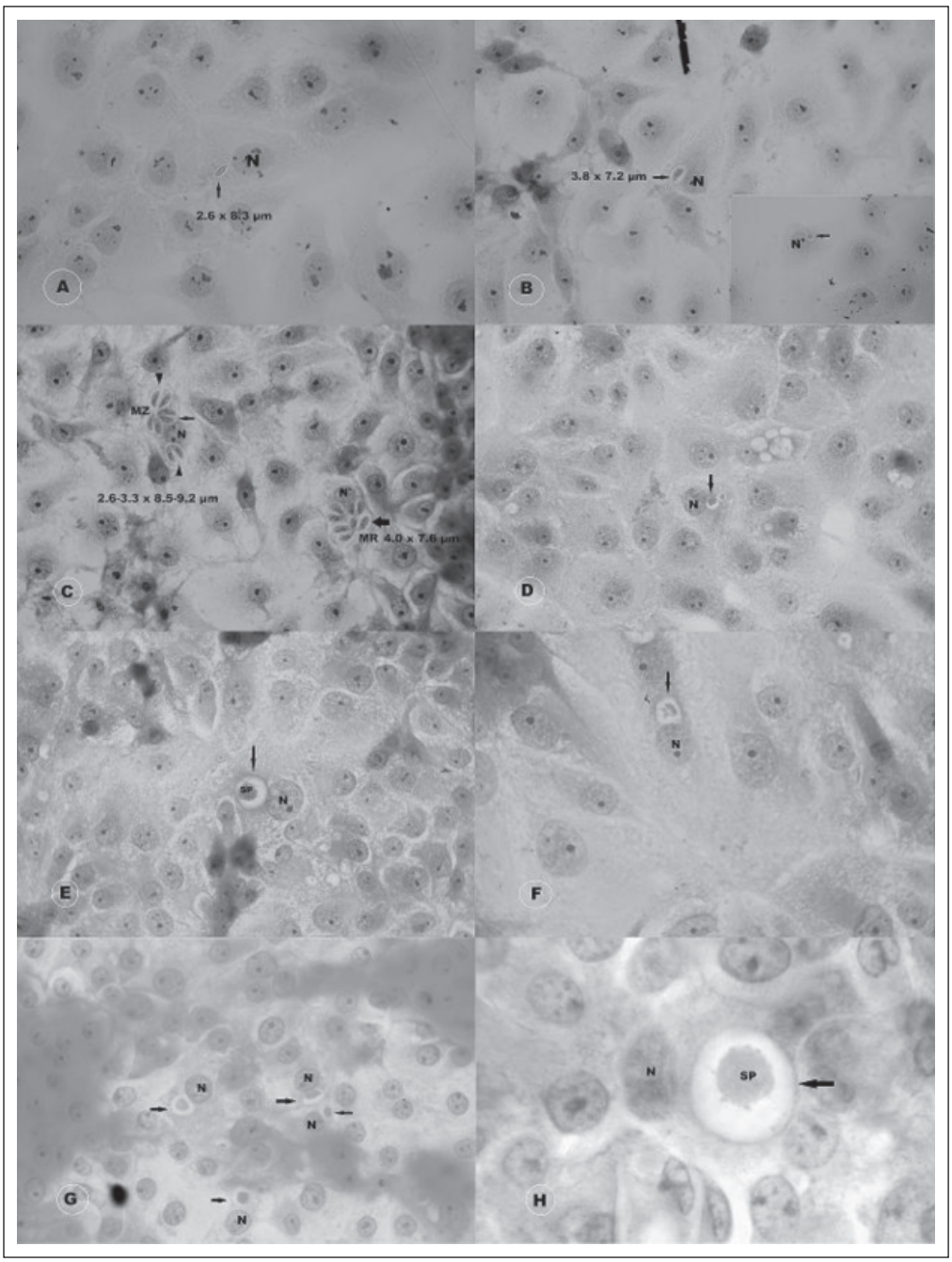

Figure 1. *In vitro* infection and development of *C. suis* in SK–6 cell line, H&E staining. (A) penetrated sporozoite (arrow) in host cell at 2 hpi, (B) shrunken and rounded intracellular penetrated sporozoite in parasitophorous vacuole at 1 dpi, (C) presumed type I merozoites formed 2 (arrow head), and 4 (thin arrow) zoites, and a presumed type I bi-nucleus meront (thick arrow) formed 8 zoites, at 3–5 dpi, (D) intracellular developing oocyst at 5 dpi showing sporonts within a thin oocyst wall, (E) intracellular mature oocyst formed through 7–14 dpi, (F) recycling of merozoites after having burst from previously infected host cells become elongated and crescent-shaped in a parasitophorus vacuole at 10 dpi, (G,H) a variety of stages of oocyst maturation during 5–14 dpi; N=host cell nucleus, PV= parasitophorous vacuole, SP=sporont, MZ=merozoite, MR=meront; 1A–1G were under 1 000x magnification, 1H was under 1 000x magnification with 3x digital zoom in.

first occurred at 7 dpi (Fig. 1E) and were seemingly prominent at 9–12 dpi. Variety in the morphological appearance of the underdeveloped oocysts were observed (Figs. 1G, H). This study limited the investigation period to 14 dpi and cultures were not further examined for extracellular parasites.

DISCUSSION

Among the broad variety of mammalian cell lines used as infection and development models of obligate parasites, including *C. suis*, the present study was the first to report the multiplication and development of *C. suis* cultured in the SK–6 cell system. The SK–6 cell line was presented as it has provided an excellent *in vitro* model for epithelial cell responses to swine pathogens generally, particularly viruses. This cell type also has advantages over other cell line candidates in being well established and already in routine use. This report showed the SK–6 cell line to be an *in vitro* supportive system for the complete endogenous life cycle of *C. suis*. Although the *in vitro* systems for development of *C. suis* were previously described (Fayer *et al.*, 1984; Lindsay & Blagburn, 1987), superiority in terms of supporting both asexual and sexual developmental stages was not achieved. Fayer and colleagues used embryonic bovine trachea cells, Madin-Darby bovine kidney cells, porcine kidney cells and bovine colon cells, while Lindsay and Blagburn used primary porcine kidney and primary foetal bovine kidney cells as host cells. All these cell types supported only multiplication of *C. suis* by asexual divisions, but not the sexual development stages of its life cycle. Over the last two decades, an *in vitro* complete endogenous development system was reported with no effective applicability (Lindsay *et al.*, 1998). The present report promotes the SK–6 cell line as a proper *in vitro* system able to support the complete endogenous life cycle of *C. suis*, conforming to its *in vivo* development found in both experimental and natural cystoisosporosis (formerly isosporosis) (Lindsay *et al.*, 1980; Harleman & Meyer, 1984). However, this study was limited to the use of purified sporozoites which were prepared from pooled oocysts of only one strain of *C. suis* for the experiments. Recently, the intestinal porcine epithelial cell line, IPEC–J2 was similarly described as an *in vitro* model for complete development of *C. suis* (Worliczek *et al.*, 2013). For asexual developmental stages, this report did not unambiguously evaluate the distinct types of meronts and merozoites because a clear identification based on measurements *i.e.* shape, length, width, group size, and number of meront nuclei (Lindsay *et al.*, 1983; Lindsay & Blagburn, 1987) was not possible. The observed meronts and merozoites were presumed as type I base on their apparent size and shape. Although asexual developmental stages were not clearly investigated, the sequence of stages was seemingly comparable with the *in vivo* reports. This chronological replication was also previously observed in most other supportive cell types. The development of sexual stages found in the studies also chronologically coincided with that of *in vivo* infections (Lindsay *et al.*, 1980; Harleman & Meyer, 1984). This might be a transcendent advantage of the SK–6 cell type over the IPEC–J2 cell system with which the latter showed significant delay in development of sexual stages compared to *in vivo* occurrence (Worliczek *et al.*, 2013). In this study intracellular oocysts first occurred at 5 dpi. In comparison in infected piglets the mature sexual stages were seen at 5–6 dpi (Lindsay *et al.*, 1980; Harleman & Meyer, 1984). However, oocysts were not detected before 9 dpi for the IPEC–J2 cell system. In comparing with *in vivo* infection, the experimental infected piglets excreted oocysts at 5–8 dpi (Mundt *et al.*, 2006; Joachim *et al.*, 2014).

For ability of sporozoites to infect the host cells in this study, the initial infectivity rate was assumed to be low in concurrence with a previous report stating that *C. suis* sporozoites possessed relative low infectivity rate, around 0.18 to 1.9%, *in vitro* (Worliczek *et al.*, 2013). Compared to other coccidian parasites that showed higher infectivity rates, *e.g.* up to 52% for *Toxoplasma gondii* (Nakao

& Konishi, 1991) and up to 18.9% for *E. bovis* (Hermosilla *et al.*, 2002), this may be considered a disadvantage of *in vitro* systems for supporting *C. suis* development. Nonetheless, the system using SK–6 cells is going to be developed to improve its efficiency and reproducibility. The development of *C. suis* in this study was limited to intracellular stages and was endogenously terminated, therefore, sporulation ability of the obtained oocysts and its infectivity to host cells *in vitro* and *in vivo* has to be investigated further.

In conclusion, the presented *in vitro* system using SK–6 was able to support complete endogenous development of *C. suis* that was comparable to its *in vivo* life cycle. Furthermore, this provides an appropriate model for the study of a vast array of aspects relating to the coccidium, especially for applications with regard to the sexual development of *C. suis*.

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