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Using pig biliary system, in vivo propagation of *Enterocytozoon bieneusi*, an AIDS-related zoonotic pathogen

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A microsporidian parasite *Enterocytozoon bieneusi* is the most common microorganism recognized in AIDS patients, and slow scientific progress is attributed to our inability to propagate the parasite. We report upon the development of a system of propagation using the pig biliary system. The parasite spores were continuously detected in the bile samples post onset of spore shedding in the gall bladder, which suggests that this organism maintain persistent infection in the biliary system and that the hepatobiliary tree may represent a reservoir of infection. In conclusion the biliary tree is an adequate niche for the propagation of *E. bieneusi*. This work has also resulted in the development of a procedure of ultrasound-guided cholecystocentesis for aspirating biles. This is a simple and non-surgical procedure, and creates no signs of clinical complications in the livers and the gall bladders after dozens of separate attempts. Thus, this is a very useful and safe technique for the aspiration of bile from live animals.

Key words: *Enterocytozoon bieneusi*, propagation, immuno-suppression, biliary system, cholecystocentesis.

Introduction

Microsporidia are obligate intracellular protozoan parasites that cause opportunistic infections in animals and humans, especially in AIDS patients. They are sufficiently unique to be classified in a separate phylum. The phylum Microspora contain nearly 100 genera and more than 1000 species of microsporidia that infect a wide range of invertebrate and vertebrate hosts [4]. These organisms are defined by a nucleated sporoplasm, a coiled polar tube, an anchoring disk, and the absence of several eukaryotic organelles, such as, mitochondria, Golgi membranes and eukaryotic ribosomes [15, 29]. Microsporidian species infecting animals and humans measure approximately 1.0 to 2.0 by 1.5 to 4.0 μm and are easily misidentified as bacteria and small yeast [4, 24]. Diagnosis of microsporidiosis can be made by detecting spores in fecal samples with trichrome, brightening, or fluorescent stains [6, 8, 28]. Species identification is usually performed by these chemical methods in conjunction with molecular assays, such as PCR [14, 17].

Several species are becoming increasingly recognized in association with significant diseases among AIDS patients. *Enterocytozoon bieneusi* is the most common microsporidium associated with AIDS. This species primarily infects enterocytes of the small intestine and causes chronic diarrhea [3, 13]. *Encephalitozoon intestinalis* and *Encephalitozoon hellem* both cause diarrhea, sinusitis, nephritis, pneumonia, and keratitis [20, 24, 29]. *Encephalitozoon cuniculi, Vittaforma corneae*, *Nosema ocularum*, and *Pleistophora* spp. have been detected less frequently in patients [2, 5, 7, 9]. The prevalence of microsporidial infection as a cause of HIV-associated diarrhea is uncertain. Since *E. bieneusi* was first recognized in biopsy specimens in persons with AIDS in 1985 [13] this parasite has been identified in 30 to 50% of AIDS patients with chronic diarrhea and also causes significant wasting and malabsorption [18, 25, 29]. Moreover, *E. bieneusi* has been recently reported to be associated with hepatobiliary and pulmonary infections and to cause papillary stenosis, acaculous cholecystitis, bile duct dilatation and sclerosing cholangitis [1, 19, 22, 30]. The sources of the microsporidia infecting humans and their transmission routes are not clearly defined. Animals are, however, the most likely source of human infections as this organism is released into the environment via animals stools, urine, and respiratory secretions. Since the detection of *E. bieneusi* in fecal samples of pigs was described in 1996 [11], the occurrence of *E. bieneusi* in several other animals such as, dogs, cats, rabbits, monkeys and cattle has been reported [10, 17, 18, 23]. At least one animal species infected with *E. bieneusi* experimentally exhibited similar clinical signs to human infection [26].

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Two monkeys immunosupressed by simian immunodeficiency virus were inoculated with *E. bieneusi* spores from an AIDS patients. Both animals began shedding spore within a week post-inoculation. One monkey became wasted and developed AIDS-related illness, and the other one developed acute septicemic illness and was near death. *E. bieneusi* from AIDS patients and from macaques monkeys with AIDS were also successfully transmitted to immunosuppressed gnotobiotic piglets [16]. Epidemiologic research on animals is critically important to clearly illustrate human infection sources to protect public health.

Despite the relatively common occurrence of *E. bieneusi* infections in human AIDS patients and the serious diseases caused by this parasite, broad spectrum studies on the organism has been limited. Thus, available information is largely circumstantial and based on limited studies in humans. The main reason for slow progress on the parasite research is the short supply of the organisms for investigations, due to inability to cultivate *E. bieneusi,* although a short term (about 6 months) in vitro culture using human lung fibroblasts and Vero monkey kidney cell lines yielded low numbers of *E. bieneusi* spores [27]. Investigations are largely dependent on the organisms purified from feces, which probably contain yeast and bacterial contaminants. One of the reasons to develop animal models is to propagate organisms using the animals. Two animal models using pigs and monkeys have been used to establish modes of transmission and persistent infection of *E. bieneusi* [16, 26]. The pig is relatively inexpensive and feasible to convenient husbandry. This animal also has a size advantage compared to the monkey and should yield high numbers of organisms. In a recent report polymorphism analysis within and between humans, pigs, cat and cattle indicated a close relationship between *E. bieneusi* strains from humans and pigs [23]. This result suggested that pigs provide a plausible source of human *E. bieneusi* infections and that the pig is an adequate model for the propagation of the organism in order to study human infection. In this study using the pig biliary system, we have developed a new model for in vivo cultivation of *E. bieneusi* to provide a source of pure parasites

Materials and Methods

Animals and procedure

Six weaned, 4 week old, healthy Yorkshire piglets from the same litter were used for this experiment and were maintained in experimental isolators with environments which were as clean as possible for the duration of the study. They were fed water and commercially available nutrient-balanced diet. Two of the animals were immediately started on a course of immunosuppressive therapy for the first four weeks, which included a daily oral dose of 15 mg/kg of cyclosporine solution (Sandoz Pharma LTD, Basel, Switzerland), and a daily intramuscular administration of 25 mg/kg of methylprednisolone sodium (Upjohn, Kalamazoo, Michigan, USA). Blood was drawn at the end of the immunosuppression regime from the necks of these two animals and one piglet was employed as a normal control for proliferative assays on B and T lymphocytes. Three of them, including one immunosuppressed, were orally inoculated with 5×10^3 to 10^5 spores of *E. bieneusi* per animal, suspended in 2 ml of PBS. The other three were inoculated directly into the gall bladder by percutaneous cholecystocentesis, described below, with the same number of spores. All six animals were orally reinoculated with the same number of spores nine weeks after the first inoculation. Animals were monitored weekly for symptoms, changes in body weight, and shedding of spores in the feces and bile. The shedding of *E. bieneusi* spores was detected with modified trichrome stain and by microscopical examination, and was confirmed by PCR amplification with the specific primer sets, as described below**.**

Analysis of immunosuppression

The ability of peripheral blood lymphocytes to proliferate in vitro was assessed by determining the response to T cell mitogen concanavalin A (Con A), and to the B cell mitogen lipopolysaccharide (LPS). 10 ml of heparin-treated blood was lysed using of 5 ml of ACK lysing buffer (0.15 M NH_4Cl, 1.0 M $KHCO_3$, and 0.01 M Na_2EDTA, pH 7.2). Lymphocytes were resuspended in DMEM-5 media, and in a 96 well plate, 4-8 $\times 10^5$ lymphocytes were then added to each well of three controls, three Con A's, and three LPS's. 50 ul of DMEM-5 medium for control well, 50 ul of 1 ug/ml Con A and 50 ul of 100 ug/ml LPS were mixed with the lymphocytes in each designated well, and the plate then incubated with seal at 37°C. After 24, 48, and 72 hours of incubation, 1 uCi of [methyl-^{3}H]thymidine was added to each well. The proliferative activity of the lymphocytes was measured using a Beckman LS 6000SE scintillation counter.

Procedure of cholecystocentesis

Animals were fasted for 12-18 hours prior to each cholecystocentesis procedure and then anesthetized by an intramuscular administration of ketamine HCl (10 mg/kg), butorphanol tartrate (0.1 mg/kg), and medetomidine (0.1 mg/kg). The hair over the abdomen was shaved and the skin prepared for aseptic surgery. Animals were positioned in dorsal recumbency, and the gall bladder visualized by ultrasonography as a hypoechoic area in the upper right quadrant of the abdomen, just caudal to the xiphoid process (Figure 1). A 20-gauge 4 cm needle was guided to the gall bladder through the hepatic parenchyma. The needle was advanced caudodorsal at an angle of 30 to 50

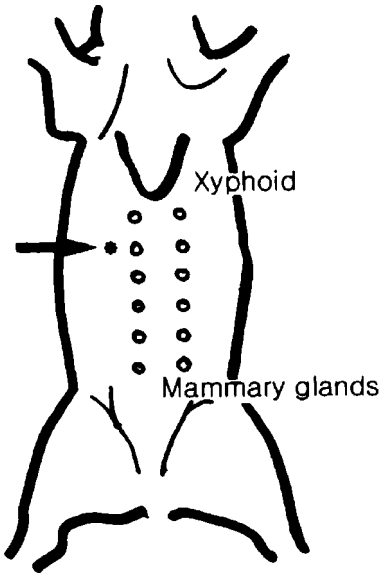

Fig. 1. Arrow indicates the site of ultrasound probe application to locate the gall bladder in dorsal recumbency. Percutaneous cholecystocentesis was performed by using a 20-gauge 4 cm needle guided by an ultrasound probe at an angle of 30 to 50°.

degrees to the ultrasound probe, until the tip of the needle was visualized on the ultrasonograph display. The tip of the needle was then advanced until it reached the surface of the gall bladder, and directed through its wall, using a controlled, quick, piercing action, and approximately 3 ml of bile was aseptically aspirated.

Light-microscopical detection of the parasites

Detection of *E. bieneusi* spores in fecal and bile materials was performed by the modified trichrome stain method [28]. Slides for light microscopical examination of stools and bile were prepared from 10-ul aliquots of a suspension of samples in 10% buffered formalin (1 : 3 ratio). Smears were fixed in methanol for 5 min and stained for 10 min at 56°C with the modified staining solution containing, 6 g of chromotrope 2R (Harleco, Gibbstown, NJ, USA), 0.15 g of fast green (Allied Chemical and Dye, New York, USA), 0.7 g of phosphotungstic acid, 3 ml of glacial acetic acid, and 100 ml of dH_2O. After staining slides were destained in acid alcohol solution (4.5 ml of acetic acid: 999.5 ml of 90% ethyl alcohol) for 10 sec and then rinsed briefly in 95% alcohol. Smears were then successively dehydrated in 95% alcohol for 5 min, 100% alcohol for 10 min, and xylene for 10 min. Slides were read under light microscopy at 1000 times magnification.

DNA extraction for PCR

Approximately 200 ul of feces was transferred to a 2 ml screw cap conical tube containing 200 ul of 0.5 mm glass beads (Biospec Products, Inc) and 400 ul digestion buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-Cl,[pH 8.0], 1% SDS, and 100 ug/ml proteinase K). The sample was then placed in a mini-bead beater at 5000 rpm for 2 minutes and incubated for 1 hour at 50°C. Samples were spun in a micro-centrifuge for 2 min at top speed. The supernatant was transferred to a new tube and mixed with an equal volume of phenol/chloroform. 300 ul of supernatant was then added to 50 ul of 5M NaCl and the mixture incubated for 10 min at 65°C. The solution was then extracted with an equal volume of chloroform and the DNA recovered from the resulting supernatant using the Geneclean system (BIO101, La Jolla, Calif., USA) following manufactures protocol for liquid samples. The DNA was resuspended in 20 ul of TE and 1 to 2 ul of the DNA solution used as PCR template.

Bile drawn by cholecystocentesis was centrifuged at 10 k rpm for 10 min. and resuspended in 1/10 volume of PBS. 10 ul of PBS-resuspended bile was applied to ISO Code Dipstiks (Schleicher & Schuell, Dassel, Germany) and dried at room temperature for 12-18 hours. The dipstiks were rinsed with 500 ul of dH_2O in a 1.5 ml tube by pulse vortexing twice for 5 seconds. 50 ul of dH_2O was then added to the dipstiks in a new tube and the tube heated to 95-100°C for 30 min. 1 to 2 ul of the DNA eluted solution was used as PCR template.

PCR amplification

The presence of spores in feces and biles was also confirmed by nested PCR amplification. The first PCR amplification was performed using the primers EBIEF1 and EBIER1, as described by De silva [12], using 45 cycles of, 94°C for 30sec, 55°C for 30sec and 72°C for 40sec. Nested amplification was performed using the primers EBIEF5: 5'-GCGACACTCTTAGACGTAT-3' and EBIER6: 5'-TGGCCTTCCGTCAATTTC-3', and conditioning by 30 cycles of, 94°C for 30sec, 57°C for 30sec and 72°C for 30sec. These primer pairs were based on nucleotide sequence of the *E. bieneusi* small subunit-ribosomal RNA. Amplification of *E. bieneusi* templates with the nested primer pair results in a 200-bp DNA fragment. Positive controls used in all experiments included the DNA of the cloned *E. bieneusi* SSU-rRNA coding region. Amplified products were eletrophoretically resolved on a 2% agarose gel and stained with ethidium bromide for visual analysis.

Results

Cholecystocentesis

The bile-filled gall bladder was readily visualized in the 4 week piglets as a hypoechoic structure, with a horizontal axis (width) of approximately 2 cm and vertical axis (length) of approximately 1.2 cm (Figure 2) and which gradually enlarged with the lapse of time. The gall bladder was located 1.5 to 3 cm deep at the abdominal midline in

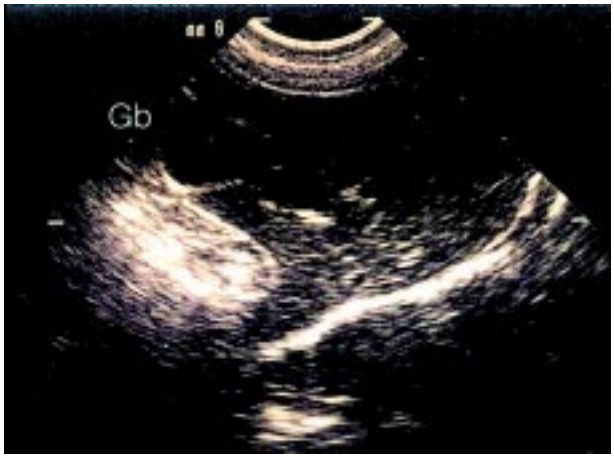

Fig. 2. Ultrasonography of gall bladder (Gb). Top of the image represents the abdominal surface. The gall bladder was visualized as a hypoechoic structure after animals were fasted for 12-18 hours prior to cholecystocentesis.

Table 1. Analysis of proliferative T(Con A) and B(LPS) cell responses of peripheral blood lymphocytes of two immunosuppressed piglets compared with a normal control

	Proliferative responses (cpm ± SD)		
Piglet	24 hours	48 hours	72 hours
Normal animal (Piglet 5)			
Control	1,172±25	1,370±157	1,446±417
Con A	47,788±5,744	64,668±1,946	1,485±211
LPS	2,037±247	1,788±90	60,309±2,600
Immunosuppressed (Piglet 3)			
Control	448±105	517±127	520±147
Con A	4,716±339	7,544±461	6,018±555
LPS	624±158	554±47	474±77
Immunosuppressed (Piglet 4)			
Control	219±66	432±79	389±64
Con A	342±6	2,064±1,122	3,578±345
LPS	288±77	182±56	368±27

piglets between 4 and 12 weeks old. Procedure technique improved with experience, eventually the centesis procedure was completed in less than 1 min in an anesthetized animal. Difficulty in aspirating bile samples was encountered at the beginning of the study in several animals whose gall bladders were too small. Appearances and weight gains of all animals were relatively normal except for one of the immunosuppressed animals which exhibited inactivity, occasional diarrhea and a significant retardation of weight gain. On the ninth week this animal had a body weight of 20 kg, compared to a 38.5 kg average, and was euthanized. Whether this was directly due to *E. bieneusi* infection or a consequence of immunosuppression must be determined in future experiments. No signs of complications due to the procedure were observed in any of the animals during or after the procedures. Occasionally slight hemorrhage occurred at the site of the skin puncture as the needle was withdrawn, and some bile samples were blood-tinged. During necropsy at the end of the experiment, gross peritoneal changes were not observed in any of the animals. Animals had small fibrous spots on the liver at the puncture sites, but no evidence of severe hemorrhage of the liver or gall bladder. Gall bladders had mild cholecystitis and fibrosis possibly due to *E. bieneusi* infection.

Immunosuppression

To investigate whether immunosuppression was necessary to mediate secure infection and propagation of *E. bieneusi* in pigs, two of the animals (piglet 3 and 4) were chemically treated as described above. The effect of the chemical regime on immunosuppression was evaluated by the proliferative response. The chemically treated piglets were severely immunosuppressed compared to the normal piglets (Table 1). The T cell proliferative activities of piglet 3 were approximately 10-folds lower than the normal animal at 24 and 48 hours. Those of piglet 4 were even much lower at 24 and 48 hours, while there were no significant differences of the activities observed at 72 hours. On the other hand, The B cell proliferative activity of the normal animal dramatically increased at 72 hours, while those of the treated animals remained low level.

Propagation of *E. bieneusi* in pigs

The detection of *E. bieneusi* parasites in feces and bile by the modified trichrome staining method and PCR amplification was used to determine the propagation of *E. bieneusi* in the animals. Spores were detected in the feces of only two animals (piglet 1 and 4) during the first week following inoculation. All 6 challenged animals, however, eventually became infected with *E. bieneusi* regardless of immune status (Table 2). Immunosuppressive treatment (piglet 3 and 4) did not significantly lead to an earlier onset of spore shedding in feces and bile. Piglets 1, 2 and 6 exhibited earlier shedding in feces than the other animals but the shedding of *E. bieneusi* in the bile of these animals did not occur earlier. In general, the onset of spore shedding in feces preceded compared to that in biles. The earliest onset of shedding in bile samples (piglet 2) was the fifth week post first inoculation while the parasite was shed in feces at the beginning of the experiment. Parasitic spores shed into biles usually became detectable between the ninth and twelfth week of the experiment (noticeably post second inoculation) except in piglet 2. Once piglets began shedding parasites into the bile, they continued to do so until the end of experiment, In addition, the amount of spore shedding in bile became considerably higher toward

Table 2. Summary of *Enterocytozoon bieneusi* propagation in piglets

Piglet	Week	1	2	3	4	5	6	7	8	9[b]	10	11	12	13
1	Feces	+	-	+	+	+	+	+	+	+	-	+	-	+
	Bile	ND	-	-	-	-	-	-	-	-	-	-	+	+
2	Feces	-	-	+	+	-	+	+	-	+	+	+	-	+
	Bile	-	-	-	-	+	+	+	+	+	+	+	+	+
3[a]	Feces	-	-	-	-	+	-	-	+	+	+	-	+	-
	Bile	ND	-	-	-	-	-	-	-	-	+	+	+	+
4[a]	Feces	+	-	-	-	+	-	-	+	+	+	ND	ND	ND
	Bile	-	ND	-	-	-		-	-	+	+	ND	ND	ND
5	Feces	-	-	-	-	-	-	-	+	+	-	+	-	+
	Bile	ND	-	-	-	-	-	-	-	-	+	+	+	+
6	Feces	-	+	+	+	-	+	+	+	-	+	+	+	-
	Bile	-	-	-		-	-	-	-	-	-	+	+	+

Piglets 1, 3, 5 were orally inoculated with 5×10^3 to 10^5 *E. bieneusi* spores, and piglets 2, 4, 6 were inoculated into gall bladder by cholecystocentesis.
+ represents presence of *E. bieneusi* parasite in the samples by modified trichrome stain method and PCR amplification.
- represents absence of the parasites.
[a] Piglets immunosuppressed with cyclosporine and methylprednisolone
[b] *E. bieneusi* spores were reinoculated orally at the designated time.
ND, Not done

the end of the experiment, while parasites were not consistently detected in feces. All animals were euthanized on the thirteenth experimental week.

Discussion

This study involves the development of a new model of propagation of an AIDS-related protozoal parasite, *Enterocytozoon bieneusi* using in vivo cultivation techniques utilizing pig biliary system. Continuous propagation of *E. bieneusi* has not been previously achieved. Preliminary data indicate that only short-term cell culture of up to six months has been accomplished previously, and this method yielded a low number of *E. bieneusi* spores [27]. Little progress has been made on either the parasite or on the nature of the diseases that it induces. Available information is largely circumstantial and based on limited direct studies in humans. Tardy progress is mainly due to an inability to cultivate *E. bieneusi*, which has markedly curtailed laboratory investigations on this organism, and on many aspects of the host-pathogen interaction. The development of a suitable model for *E. bieneusi* propagation has been identified as a research priority. Our effort reported here, to develop a model led to the successful propagation of the parasite by persistent infection in the pig biliary system. This organism is found in enterocytes and in the cells of the lamina propria, and has recently been described in epithelial cells of the hepatobiliary tree [1, 19, 22]. In a report attempting to localize the site of persistent *E. bieneusi* infection in immunologically normal rhesus macaques, 31 animals underwent endoscopic examination and biopsy of the duodenum and proximal jejunum [17]. 27 of these animals also underwent examination of the hepatobiliary tree. No case of *E. bieneusi* was found in the sampled sections of intestine from the normal monkeys. In contrast, PCR performed on DNA isolated from bile was positive in several normal animals with *E. bieneusi* DNA detected in feces. This indicated that intestines in normal animals do not allow persistent *E. bieneusi* infection. This may be due to the removal of parasites by host clearance mechanisms such as gut immunity and intestinal peristalsis. The parasites primarily infecting in enterocytes are likely to have migrated to the biliary system and established persistent infection in hepatobiliary tree, and the hepatobiliary tree may represent a reservoir of infection. This study also shows a similar pattern. Spores were detected in feces earlier than in biles, however, detection in feces was not consistent with the presence of spore in the biliary system. These results enable us to conclude that the biliary tree is an adequate niche for the propagation of *E. bieneusi.*

The contribution of the hosts immune status to inhabitation capacity and pathogenesis remains unclear. This organism, however, causes serious diseases mainly in immunodefiecient individuals, which suggests that a suppressed level of host immunity plays a major role in inducing disease. Therefore, long term immunosuppression in animals may cause severe illness that may lead to deaths. The chemical immunosuppression regime in this study involving cyclosporine and methylprednisolone sodium severely immunosupressed the animals. However, it did not necessarily lead to earlier onset of spore shedding or better propagation. These results suggested that in vivo propagation of *E. bieneusi* spores in pigs may not need the suppression of host immunity. Non-immunosuppression may be a better strategy for the propagation, as this will eventually generate higher number of the spores and prolong the life span of the animals.

During the course of this study we also developed a procedure for transhepatic ultrasound-guided cholecystocentesis for use in the bile sampling of pigs. Aspirating biles by percutaneous cholecystocentesis with ultrasonography to examine *E. bieneusi* infection was first described in monkeys [17]. Our technique should be very useful for the investigation of other infections and the determination of the chemical status of bile, since techniques of sampling biles involving surgical procedures increase study time and costs, and place the animals at risk. Clinical findings after dozens of separate trials suggest that the ultrasound-guided cholecystocentesis procedure is satisfactory although several pinpoint areas of hepatic fibrosis and slight hemorrhage resulting from repeated

puncturing were observed. Use of a small gauge needle to create a smaller puncture site is preferable, as trauma and the possibility of bile leakage are tempered. In pigs we found no such problems or aspiration difficulty when 20-gauge needles were used. Fasting the animals is also important for hypoechoic visualization, since fasting prior to the centesis procedure promoted gall bladder distention. Prior to the centesis procedure the animals should be adequately anesthetized. When the animals struggle against manual restraint, their gall bladders appeared to be reduced on the ultrasonographic display, and such agitation may rapidly empty their gall bladders. The combination of ketamine HCl, butorphanol tartrate and medetomidine was a very effective anesthetization regime. In conclusion, as a method for repeated bile sampling in pigs, we have found ultrasound-guided percutaneous cholecystocentesis to be rapid, minimally traumatic, and safe.

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