RESEARCH NOTE

PREVALENCE AND SPECIES IDENTIFICATION OF *CRYPTOSPORIDIUM* FROM FECAL SAMPLES OF HORSES IN TAIWAN

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Abstract. Cryptosporidiosis is a zoonotic disease caused by the protozoan parasite *Cryptosporidium*. A total of 436 horse fecal samples were collected from 19 farms, and acid-fast staining method was used for primary screening. *Cryptosporidium* oocysts were found in 161 samples, among which 33 positive sample were selected for nested PCR, restriction fragment length polymorphism analysis and DNA sequencing of 18 S rDNA, showing 31 samples to be bovine *C. parvum* and 2 *C. felis*. The methods employed in this study should be useful as tools to identify cryptosporidiosis genotypes and species of livestock.

Keywords: *Cryptosporidium*, genotype, horse, nested PCR, RFLP, Taiwan

INTRODUCTION

Cryptosporidium is a zoonotic pathogen, which can be transmitted from animals to humans or from humans to animals. *Cryptosporidium* infects a large number of vertebrates, including humans, horses, cats (*Felis catus*) and dogs (*Canis familiaris*). Livestock is often considered a source of environmental source of *Cryptosporidium* (Xiao *et al,* 2004). *Cryptosporidium* genus currently comprises at least 24 valid species and more than 40 genotypes, most of which are host-adapted and have a narrow host range (*eg*, *C. canis*, *C. felis* and *C. hominis* in dogs, cats and humans, respectively) (Thompson *et al,* 2008; Xiao and Fayer, 2008). Some species or genotypes, most notably *C. parvum* and *C. cervine*, have a broader host range that includes ruminants and humans (Xiao and Fayer, 2008; Xiao and Feng, 2008).

Cryptosporidiosis was first identified in immunocompromised Arabic foals

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and was observed to cause severe diarrhea (Snyder *et al*, 1978). *Cryptosporidium* infection in horses with normal immune function was first identified by Gajadhar *et al* (1985). Since then, there have been occurrence of *Cryptosporidium* in horses with normal immune function, suffering from similar symptoms of diarrhea (Coleman *et al*, 1989; Xiao and Herd, 1994; Netherwood *et al*, 1996; Olson *et al*, 1997). Some cases are primary infections without other enteropathies (Coleman *et al*, 1989). Xiao and Herd (1994) reported the rate of positive *Cryptosporidium* infection in horses with diarrhea is 54% and in horses without diarrhea, *ie*, having no clinical signs, 14% (Xiao and Herd, 1994).

Polymerase chain reaction (PCR) method has been used to identify pathogenic *Cryptosporidium* spp (Laxer *et al*, 1991). Although many sequences of *Cryptosporidium* spp have been reported, but *Cryptosporidium* spp infection in horses was not identified (Morgan and Thompson, 1998; Sulaiman *et al*, 1998; Bornay-Llinares *et al*, 1999) until the nucleic acid sequence of *Cryptosporidium* in horses was first reported by Ryan *et al* (2003). Cryptosporidiosis in horses has been identified as *C. parvum* by oocyst morphological characteristics, but it is important to use molecular biology techniques to be able to more accurately classify the species and to understand its role in human public health.

The role of horse-infected *Cryptosporidium* in zoonotic diseases is unknown. The *Cryptosporidium* animal manager survey conducted by Mahdi and Ali (2002) indirectly showed that horses may be a significant source of zoonotic infection, and moreover, no information is available regarding *Cryptosporidium* epidemiology in horses in Taiwan. Thus, it is necessary to determine the prevalence of *Cryptosporidium* infection in horses in Taiwan.

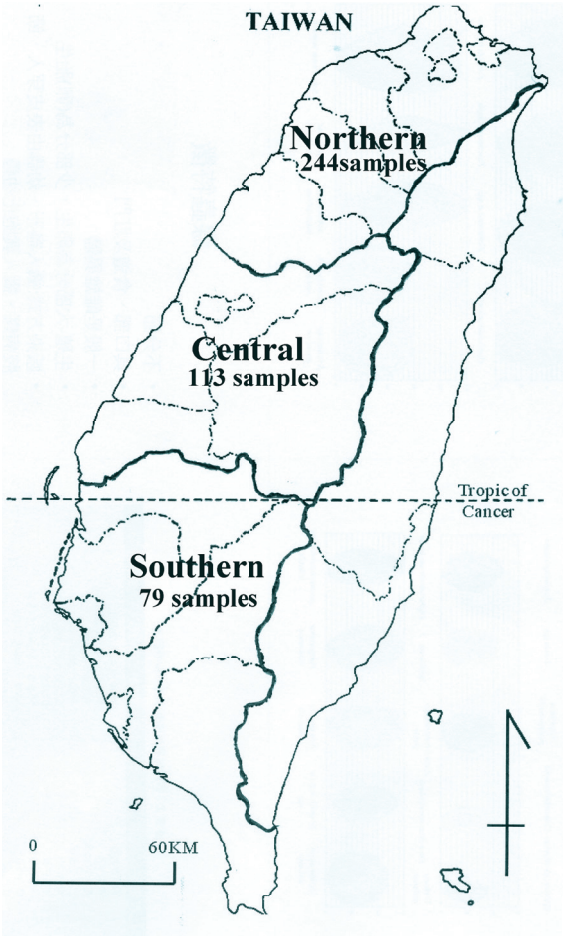

Fig 1–Map of Taiwan showing the sites where fecal samples of horses were taken.

MATERIALS AND METHODS

Horse fecal samples

Fresh fecal samples from 436 horses at 19 ranches (10 in northern, 5 in central and 4 in the southern Taiwan) (Fig 1) from September 2001 to October 2003 were mixed with 2.5% potassium dichromate solution in a ratio of 1:1 and stored at 4ºC. Samples were washed in 0.85%

Table 1
Cryptosporidium infection in horses in Taiwan.

	Northern Taiwan	Central Taiwan	Southern Taiwan	Total
No.	244 (10 ranches)	113 (5 ranches)	79 (4 ranches)	436 (19 ranches)
Positive	93	36	32	116
Infected rate	38%	32%	40%	37%

normal saline before microscopic examination and DNA extraction. Positive control was *C. parvum* oocysts from a sick sheep at Taihung, Taiwan and a hamster subculture from our laboratory, which was confirmed the infection by acid-fast staining, PCR and gene sequencing. The nucleotide sequence of the positive control was determined using the ABI DNA automatic sequencer (Model 3730; Applied Biosystems, Foster City, CA) and accession number: AF093490.

Microscopic examination

Stool consistency of all samples was recorded, and samples were checked for the presence of *Crytosporidium* oocysts under a light microscopy (200x magnification). Each sample was examined using a modified Ziehl-Neelsen acid-fast stain for *Crytosporidium* oocysts according to Henriksen (1981).

PCR identification

DNA was extracted from oocysts using an UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) and stored at -20ºC prior to analysis. The 18 S rDNA of *Cryptosporidium* was amplified using nested PCR (Xiao *et al*, 1999). PCR amplicon (834 bp) was first generated using primers All-1 (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3') and All-2 (5'-AAG GAG TAA GGA ACA ACC TCC A-3', and subsequently an amplicon of 585 bp using primers 18SiCF1 (5'-CCT ATC AGC TTTAGA CGG TAG-3') and 18SiCR1 (5'- TCT AAG AAT TTC ACC TCT GAC TG-3') (Ryan *et al*, 2003). Thermocycling conditions of PCR (Bio-Rad Laboratories, Hercules, CA) were as follows: 94ºC for 5 minutes; 35 cycles of 94ºC for 45 seconds, 59ºC (for the first PCR) or 56ºC (for the second PCR) for 45 seconds, 72ºC for 60 seconds; followed by a final heating at 72ºC for 10 minutes. An aliquot of the PCR solution was digested for 2 hours at 37ºC with 10 U *Xap*I, *Vsp*I and *Dra*I (Gibco/Life Technologies, Grand Island, NY). Undigested controls and digested samples were separated by 2% agarose gel-electrophoresis at 100 V for 0.5 hour in buffer containing ethidium bromide (0.5 μg/ml) and visualized under UV light.

DNA sequence analysis

PCR amplicons were sequenced by Tri-I Biotech, Taiwan, and the sequences were analysed using BioEdit software (Hitachi Software Engineering, Tokyo, Japan; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). These sequences (samples 1-33) were 99.7-100% identical with GenBank accession number AF093490 or AF108862.

RESULTS

Red or pink oval oocysts measuring 3-6 μm were observed in 161 (37%) cryptosporidiosis-positive specimens of 436 samples, with positive rate in northern,

central and southern Taiwan of 38 (244 specimens), 32 (113) and 40% (79), respectively (Table 1).

Oocysts from 33 samples (containing an average of >100 oocysts per 5 fields) were subjected to PCR and DNA sequencing analysis. Nested PCR of all samples revealed the presence of 585 bp amplicons, which were digested by *Vsp*I into 530 bp and 55 bp fragments (data not shown) indicative of either bovine *C. parvum* or *C. felis*. The 605 bp fragment of *C. felis* can be digested by *Dra*I into 2 fragments of 361 bp and 244 bp, which was obtained in 2 samples, which were considered as *C. felis* (data not shown).

DNA sequence analysis of the putative 31 bovine *C. parvum* 585 bp fragment showed 100% identity with bovine *C. parvum* standard strain AF093490 from GenBank (Fig 2). The DNA sequences of the 2 putative *C. felis* samples were 99.7% identical (2 bases different) with *C. felis* standard strain in GenBank (accession no. AF108862) (Fig 2).

DISCUSSION

Previous inspection methods for horse *Cryptosporidium* included tissue slices (Snyder *et al,* 1978; Mair *et al,* 1990), acid-fast stain (Henriksen, 1981; Cole *et al,* 1998), immunofluorescence analysis (Olson *et al,* 1997; Cole *et al,* 1998), and flow cytometry (Arrowood *et al,* 1995; Cole *et al,* 1998, 1999). Because manure from herbivorous animals contains large amounts of crude fiber and impurities, we used the concentration precipitation method in order to filter out most of the plant fibers, thereby decreasing interference with microscopy and increasing the positive rate of *Cryptosporidium* oocyst detection by the acid-fast staining method. Although the sensitivity of acid-fast staining is poor and lacks specificity, it is the easiest and most effective method for veterinary clinical diagnosis. On the other hand, PCR is a very sensitive method, but requires experienced personnel and is expensive. Therefore, it is not used routinely for investigations. However, PCR is a powerful tool for identification of genotypes.

We found that the *Cryptosporidium* infection rate in Taiwanese horses was up to 37%. Based on previous studies, stallion ranches have the highest infection rate and adult horses have the lowest infection rate (Cole *et al,* 1998). Although most horses in Taiwan are adults and there are no stallion ranches in Taiwan, the positive rate is higher than that in other countries (Coleman *et al,* 1989; Xiao and Herd, 1994; Netherwood *et al,* 1996; Olson *et al,* 1997; Cole *et al,* 1998, 1999; McKenzie and Diffay, 2000). This can be attributed to the following factors: 1) most horses in Taiwanese ranches are imported trained geldings aged 5-15 years; and adult animals infected with *Cryptosporidium* generally have no clinical symptoms and the infected animals have a prototype infection that may not be noticed by their owners (Xiao and Herd, 1994; Cole *et al,* 1998), 2) Horse races are often held on various racing courses or ranches in Taiwan; and because not all ranches have specialized vehicles to transport horses, they have to rely on commercial transportation, resulting in the possibility of mutual infection among transported or race horses. 3) Horse trade between horse ranches is flourishing, and thus, commercial vehicles often shuttle between horse ranches; if these vehicles are not cleaned and sanitized, they may become mechanical carriers. Thus, it is suggested that horses that return from outdoors should be quarantined for at least 14 days and the

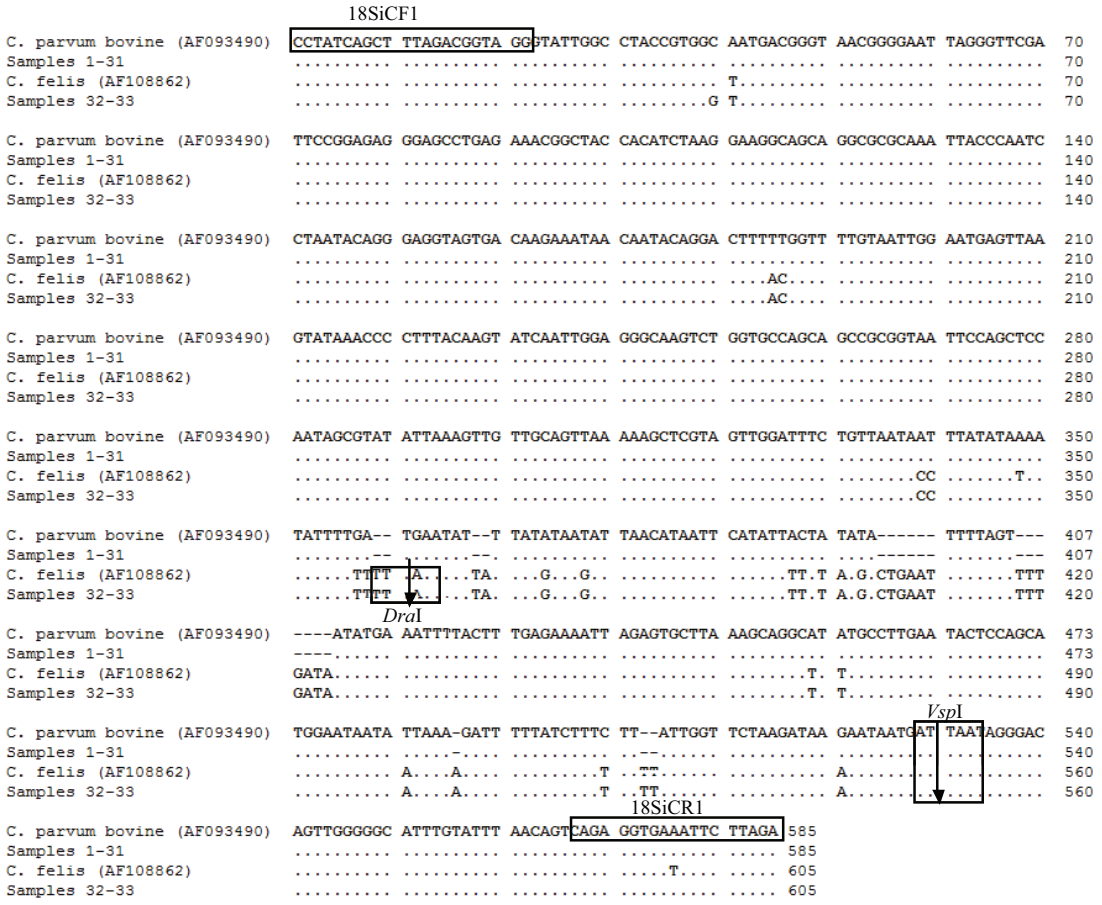

Fig 2–18S rDNA sequences of *C. parvum* bovine genotype (GenBank accession no. AF093490) and *C. felis* (AF108862). Location of PCR primers (18SiCF1, 18SiCR1) and *Dra*I and *Vsp*I sites are shown.

vehicles transporting the horses should be thoroughly cleaned and disinfected to prevent *Cryptosporidium* infection.

Little information is available regarding the epidemiology of *Cryptosporidium* in Taiwan. Previous studies investigating *Cryptosporidium* in Taiwanese water sources have mostly used specific fluorescence microscopy (Hsu *et al*, 1999a,b; Hsu *et al*, 2001) and have detected an average of 22.1 oocysts/100 liter of water sample. The *Cryptosporidium* infection rate in feeding animals in Taiwan is 32.6% in cattle (Huang *et al*, 2012) and 38% (173/460) and 36% (44/123) in cattle and goats, respectively (Watanabe *et al*, 2005). This study is the first report of *Cryptosporidium* infection rate and identification of species in Taiwanese horses. *Cryptosporidium* oocyst shedding in livestock in Taiwan is ubiquitous. As the majority of *Cryptosporidium* detected in horse feces was bovine *C. parvum*, its zoonotic potential in causing a human outbreak of cryptosporidiosis should not be ignored. Transmission of *Cryptosporidium* oocyst from animal to humans through contamination of the environment by animal feces should be

considered.

Horses infected with *Cryptosporidium* spp may have no clinical symptoms of diarrhea (Xiao and Herd, 1994). Among the horse specimens analysed in this survey, only some horses (total infection rate: 15-31%) in one ranch had diarrhea with watery stool for 10-15 days, but the remaining horses had no diarrhea, as described in the literature (Xiao and Herd, 1994).

Some horse ranches conduct horse manure reuse processing, and some ranches sell unfermented horse manure in the form of organic fertilizer (Lin, 2003). Lin (2003) found that manure from fermentation processing contains 4% detectable *Cryptosporidium* spp oocysts, which could be found in fertilizers sold in markets after traditional stack processing. If farmers use such horse manure containing *Cryptosporidium* oocysts in fields or flowerbeds, workers may become infected or agricultural products and the environment may also become contaminated. Therefore, animal wastes should only be used as organic fertilizers after thorough fermentation processing.

In this study, we used acid-fast staining to detect the positive samples of *Cryptosporidium* in horse feces, and then applied nested PCR to detect the actual species in the infected samples. DNA sequencing confirmed the putative genotypes inferred from restriction fragments polymorphism analysis. Thus these methods should be useful as a model reference in investigating *Cryptosporidium* spp or genotypes in horses.

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