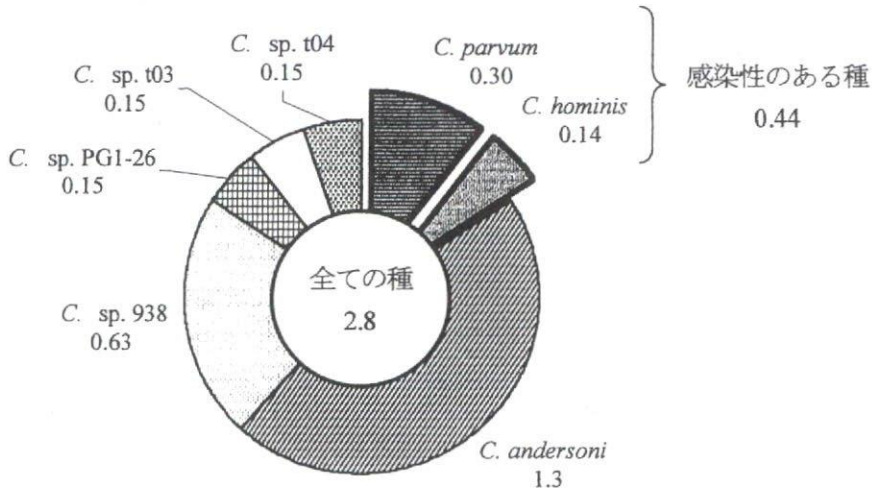

図2 小山川における種・遺伝子型ごとの存在濃度
（ポアソン分布を基にした最尤法（MPN 法）での推定結果、単位 [oocysts/100L]）

4．考察

4．1．小山川におけるクリプトスポリジウムの存在状況

本研究では、自然由来のクリプトスポリジウムの種・遺伝子型を類別する手法として、DGGE 法を初めて適用した。その結果、河川水中に存在するクリプトスポリジウムの種・遺伝子型を、精度よく検出、判別することができた。また、定量値として、検出されたクリプトスポリジウム全体の濃度だけではなく、個々の種・遺伝子型ごとに、その存在濃度を推定することができた。これにより、以下の知見を得ることができた。

小山川において、ウシに特異的に感染する *C. andersoni* が、クリプトスポリジウム全体の約半数を占めることが明らかとなった。これにより、この地域におけるクリプトスポリジウムの汚染源として、ウシが重要な位置を占めることが示唆された。

また、これまでまだ種として分類されていない遺伝子型が 4 種類（*C.* sp. 938、*C.* sp. PG1-26、*C.* sp. t03、*C.* sp. t04）検出され、濃度に換算して全体の 3 分の 1 程度を占めることがわかった。これらの遺伝子型に関しては、*C.* sp 938 がヘビ[39]、*C.* sp. PG1-26 がブタ[40]から検出されたという報告がある程度で、宿主特異性や、ヒトへの感染性に関する情報はまだ得られていない。残り 2 つ（*C.* sp. t03、*C.* sp. t04）は、これまでに報告されていない新しい種である可能性がある。さらに、同一試料に複数（最大 4 種類）の種・遺伝子型が混在していたことをあわせて考えると、小山川におけるクリプトスポリジウムによる汚染が、様々な起源により発生する複合的なものである可能性が示された。

さらに、クリプトスポリジウム症患者からの単離株の大部分を占める *C. parvum* や *C. hominis* が、河川水中においては約 16%しか存在していないことが示された。これは、クリプトスポリジウムによる水系感染症のリスクを評価する上で、重要な知見である。現在クリプトスポリジウム検出手法として広く用いられている顕微鏡観察[35,36]や、定量 PCR 法[33,42-45]では、検出されたクリプトスポリジウムの種・遺伝子型に関する情報を得ることができない。このような定量結果を基にした感染症リスク評価においては、検出されたクリプトスポリジウムが等しくヒトに対する感染性を持つ、と仮定することが多い。しかし、今回の調査において、ヒトに対する感染性を持つクリプトスポリジウムが、全体の 6 分の 1 程度しか存在しなかったことから、リスク評価に用いるデータを収集するにあたっては、定量だけではなく、DGGE 法やクローニング、RFLP 法のような、種・遺伝子型を判別する手法を組み合わせることが重要であることが示された。

4．2．クリプトスポリジウムの種・遺伝子型の判別における DGGE 法の有用性

DGGE 法の最大の利点は、本手法が遺伝子群集解析に広く適用されていることからもわかるように、多数の遺伝子配列を含む試料を、個々の遺伝子配列ごとに類別できる点にある。特に、同一の遺伝子配列が複数の試料に連続して存在する場合には、生成したバンドの位置を確認するだけで、その種・遺伝子型を判断することが可能である。また、RFLP 法と異なり、試料中に未知の遺伝子配列が含まれていた場合でも、それを既知のものと識別することが容易である。したがって、今回のように、得られる遺伝子配列が未知であるような場を対象とした調査を行う場合には、特に適した手法であると考えられる。実際、小山川における調査の結果、新しい遺伝子配列が検出され、また同一試料に最大 3 種類の種・遺伝子型が混在している場合でも、それぞれを判別することができた。

一方、DGGE 法の欠点としては、RFLP 法と比較して時間を要する点が挙げられる。しかし本研究での調査のように、迅速に結果を出すことが必要ではない場合は、この点に関しては問題ない。次に、DGGE 法により得られたバンドの位置だけでは、その遺伝子配列がどの種・遺伝子型によるものであるのかが判断できないことが挙げられる。これはすなわち後段にシーケンシングが必要である、ということを意味している。確かにシーケンシングにより最終的な結果を得るまでにはさらに時間が必要であるが、遺伝子配列を解読することで、試料に含まれる全ての種・遺伝子型の判別が可能であり、また未知の遺伝子配列を検出することも可能であることを考慮すると、より詳細な結果が得られるという点で、優れた性質であるということもできる。さらに、DGGE 法に適用できる塩基長には制限があり、最大で 500bp 程度であるとされている [41]。この点は、プライマーの設計や選択の際に、種による配列の差が十分にある領域を増幅部位とすることで解決できる。実際に本研究では、約 300bp を増幅対象としたプライマーを選択して、多くの種を類別できることを示した。

以上より、クリプトスポリジウムの種・遺伝子型の判別に DGGE 法を適用することで、先に述べた RFLP 法よりも精度が高く、またクローニングとシーケンシングを組み合わせた手法よりも経済的な調査結果を得ることができることが示された。

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Short communication

Infectivity of a novel type of *Cryptosporidium andersoni* to laboratory mice

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Abstract

Previously, we reported 'a novel type' of *Cryptosporidium andersoni* detected from cattle in Japan, and showed that the isolate was infective to mice. In the present study, we examined the patterns of oocyst shedding in both immunocompromised and immunocompetent mice, as well as pathological lesions in the infected mice. After oral inoculation with 1×10^6 oocysts, all five severe combined immunodeficiency (SCID) mice began to shed endogenously produced oocysts on day 6 post-inoculation (p.i.). The number of oocysts per day (OPD) reached 1×10^6 on day 17 p.i., and an OPD level of 1×10^6 to 10^7 was maintained until 91 days p.i. when the mice were sacrificed. In the five immunocompetent mice inoculated with 1×10^6 oocysts, the prepatent and patent periods were 6 and 19 days, respectively, and the maximal OPD level was 1.5×10^5 on average. On histological examinations of infected SCID mice, a large number of parasites were present on the surface of the gastric glands of the stomach, but not in other organs examined. In conclusion, the novel type of *C. andersoni*, which genetically coincides with *C. andersoni* reported in other countries, is infective to mice, but susceptibility was lower than that of *Cryptosporidium muris* infecting rodents from the perspective of infectivity to immunocompetent mice.

Keywords: *Cryptosporidium andersoni*; *Cryptosporidium muris*; Infectivity to mice; Pattern of oocyst shedding

Cryptosporidium muris has been found in cattle and other ruminants (Upton and Current, 1985; Anderson, 1987, 1991). It has also been reported that the isolate from cattle is genetically distinct from that from rodents (Morgan et al., 2000), and dose not infect immunocompetent or immunocompromised mice unlike the isolate from rodents (Lindsay et al., 2000; Morgan et al., 2000). Therefore, in 2000, the isolate from cattle was distinguished from *C. muris* infecting rodents, and the name *Cryptosporidium*

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andersoni was proposed (Lindsay et al., 2000). Recently, however, a *Cryptosporidium* isolate called 'a novel type' of *C. andersoni* was isolated from cattle in Japan (Satoh et al., 2003; Matsubayashi et al., 2004). The isolate was genetically identical with that of *C. andersoni* reported previously using the 18S ribosomal RNA gene sequence analysis, but was successfully transmitted to severe combined immunodeficiency (SCID) mice (Satoh et al., 2003; Matsubayashi et al., 2004). These findings showed that the isolate in Japan was biologically different from *C. andersoni* isolates in other countries. However, since then, there have not been further biological analyses of the novel type of *C. andersoni* in mice. In the present study, we examined the patterns of oocyst shedding in both immunocompromised and immunocompetent mice, as well as pathological lesions in the infected mice.

Oocysts of *C. andersoni* used in the present study were isolated from cattle in Hokkaido, Japan (Matsubayashi et al., 2004), and maintained by passage in SCID mice. Fecal pellets were collected and oocysts were purified by the sugar flotation method. Oocysts were resuspended in distilled water and stored at 4 °C for less than one month.

For experimental infections, five-week-old female C.B-17/Icr-SCID Jcl and C.B-17/Icr-+/+ mice (Clea Japan Inc., Japan) in groups of five were used. Each mouse was inoculated with 1×10^6 oocysts in 0.1 ml of distilled water by gastric intubation and separately housed in wire-bottom cages placed on a tray containing 5 mm of water to keep the feces wet. All cages were kept in an environmentally controlled room, which was maintained at 25 °C. The mice were given sterilized water and standard pellet diet (CE-2, Clea Japan Inc.). All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" by the Department of Protozoal Diseases, Graduate School of Medicine, Osaka City University. Fecal examinations for oocysts were carefully carried out by the sugar centrifugal flotation method. Briefly, samples were collected daily from 4 through 40 days post-inoculation (p.i.), and at 3-day intervals from 40 through 91 days p.i. Then, a sucrose solution with a specific gravity of 1.2 was added to the sediment of 1 g of each sample. After centrifugation, oocysts floating on the surface were recovered and put onto a glass slide. The entire smear was observed

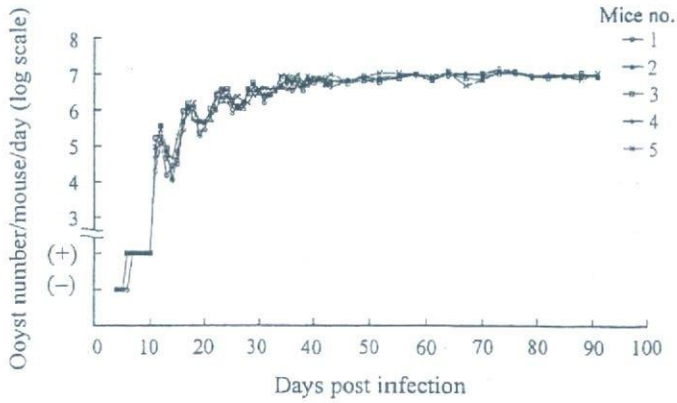

Fig. 1. Patterns of oocysts shedding in five SCID mice inoculated with 1×10^6 oocysts of the novel type of *C. andersoni*.

under a light or differential interference contrast microscopy at 200- or 400-fold magnifications. The number of oocysts per day (OPD) was estimated. The SCID mice were sacrificed on day 91 p.i., and at necropsy, esophagus, stomach, duodenum, jejunum, ileum, cecum, heart, lung, kidney, liver and bile duct were collected, and fixed with 10% neutral buffered formalin. Histological sections, stained with haematoxylin and eosin, were examined for the presence of endogenous stages of the parasite.

The patterns of oocyst shedding in SCID mice are presented in Fig. 1. A few oocysts were first detected in the feces on day 6 p.i. Countable oocysts (1×10^3 to 10^4) were found from 11 days p.i., and the number of OPD gradually increased till 40 days p.i., with the number of OPD reaching 1×10^6 on day 17 p.i. From 11 to 40 days p.i., the number of OPD slightly

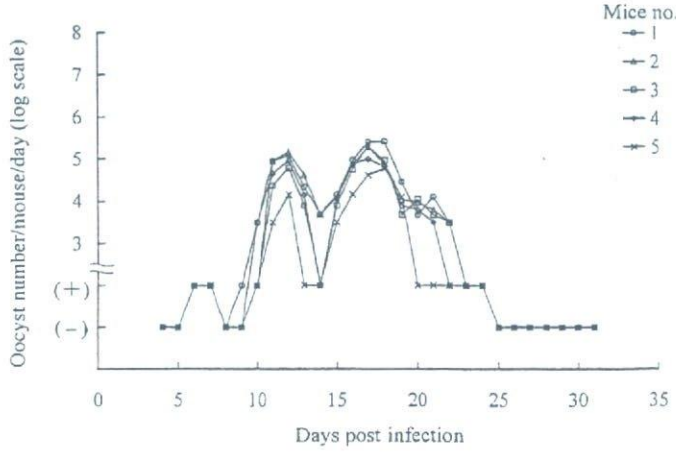

Fig. 2. Patterns of oocysts shedding in five immunocompetent mice inoculated with 1×10^6 oocysts of the novel type of *C. andersoni*.

decreased four times at 5–8 days intervals. After 40 days p.i., the average number of OPD continually remained 8.1×10^6. The maximal number of OPD was $1.1–1.3 \times 10^7$ (1.2×10^7 on average). There were no clinical symptoms such as diarrhea.

The number of OPD in immunocompetent mice is shown in Fig. 2. Small numbers of oocysts were first detected in the feces on day 6 p.i. Large numbers of oocysts were found from 10 to 11 days p.i. The number of OPD did not increase as much as that in SCID mice and the number eventually began to decrease till none could be detected. On monitored days, the number of OPD fluctuated several times at 5–7 days intervals. During the patent period of 19 days, the maximal number of OPD for five mice was $0.6–2.4 \times 10^5$ (1.5×10^5 on average).

On histological examinations of infected SCID mice, a large number of parasites including trophozoites, shizonts and oocysts were present on the surface of the gastric glands of the stomach (Fig. 3), but not in the non-glandular stomach. There was no inflammatory response noted around the parasites. There were no endogenous stages found in other organs examined.

In experimental infections of the novel type of *C. andersoni* to SCID and immunocompetent mice, both mice proved their susceptibility to the infection. Careful examination in the present study demonstrated that very few oocysts were shed from either SCID or immunocompetent mice on day 6 p.i. In SCID mice, the number of OPD gradually increased to about 1×10^7, and mice showed persistent infections. However, the number of OPD in immunocompetent mice was low, and there were no oocysts detected after 25 days p.i. These results show that the immune system plays a major role in infection of the novel type of *C. andersoni*, like other *Cryptosporidium* spp. (McDonald et al., 1992).

Genetically, *C. muris*, which is also infective to mice, is closely related to *C. andersoni*. To analyze biological differences between the novel type of *C. andersoni* and *C. muris*, we compared our results with the pattern of oocyst shedding of *C. muris* reported previously. The pattern of OPD in SCID mice in the present study was similar to that of *C. muris* in immunocompromised mice (athymic mice) reported previously (Taylor et al., 1999). However, in immunocompetent mice, the patent period of the

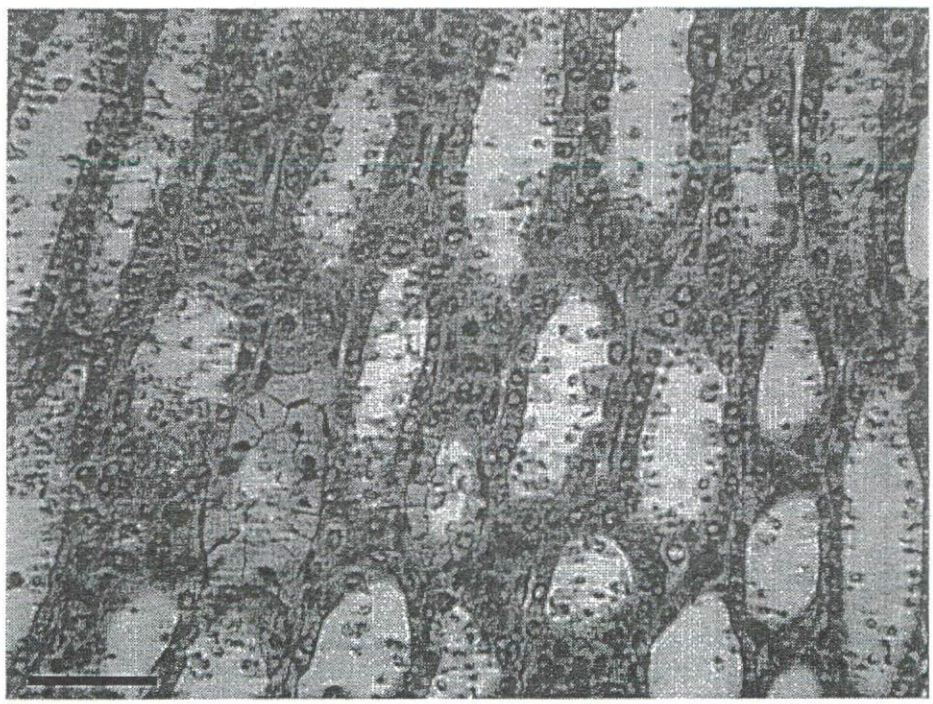

Fig. 3. Hematoxylin and eosin stained paraffin section of the stomach of SCID mouse infected with a novel type of *C. andersoni*. Surfaces of gastric glands are covered with large numbers of parasites; scale bar = 50 μm.

novel type of *C. andersoni* (19 days) was much shorter than that of *C. muris* (between 34 and 75 days in Icr strain mice), and the maximal number of OPD of the novel type of *C. andersoni* (1.5×10^5 on average) was much lower than that of *C. muris* (2.6×10^7 on average) (Iseki et al., 1989). These findings indicate that the infectivity of the novel type of *C. andersoni* to mice is lower than that of *C. muris*.

The location in mice of the novel type of *C. andersoni* was restricted in the glandular part of the stomach, which is the same as *C. andersoni* reported previously in cattle (Lindsay et al., 2000). There were no gross pathological lesions and no signs of any inflammations. These findings in mice were similar to those of *C. muris* (Iseki et al., 1989; Taylor et al., 1999).

We examined the biological features of the novel type of *C. andersoni* in SCID and immunocompetent mice. We found differences in patterns of oocyst shedding between this isolate and *C. muris* in immunocompetent mice. In conclusion, the novel type of *C. andersoni*, which genetically coincides with *C. andersoni* reported in other countries, is infective to mice, but the susceptibility of mice to *C. andersoni* is lower than that to *C. muris* in immunocompetent mice. Further studies are needed to clarify the host range of this novel type of *C. andersoni*.

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Zoonotic Genotype of *Giardia intestinalis* Detected in a Ferret

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ABSTRACT: *Giardia intestinalis* has been found in a variety of mammals, including humans, and consists of host-specific and zoonotic genotypes. There has been only 1 study of *G. intestinalis* infection in weasels, but the genotype of its isolate remains unclear. In this study, we report the isolation of *Giardia* in a ferret exhibited at a pet shop. The isolate was analyzed genetically to validate the possibility of zoonotic transmission. *Giardia* diagnostic fragments of the small subunit ribosomal RNA, β-giardin, and glutamate dehydrogenase genes were amplified from the ferret isolate and sequenced to reveal the phylogenetic relationships between it and other *Giardia* species or genotypes of *G. intestinalis* reported previously. The results showed that the ferret isolate represented the genetic group A-I in assemblage A, which could be a causative agent of human giardiasis.

The flagellate *Giardia* is a well-known intestinal parasite, which infects a wide range of vertebrate hosts, including humans. At present, 6 species in this genus, i.e., *G. intestinalis* (syn. *G. lamblia*, *G. duodenalis*) in humans, livestock, and other domestic animals, *G. microti* and *G. muris* in rodents, *G. psittaci* and *G. ardeae* in birds, and *G. agilis* in amphibians, which can be distinguished in view of the morphology

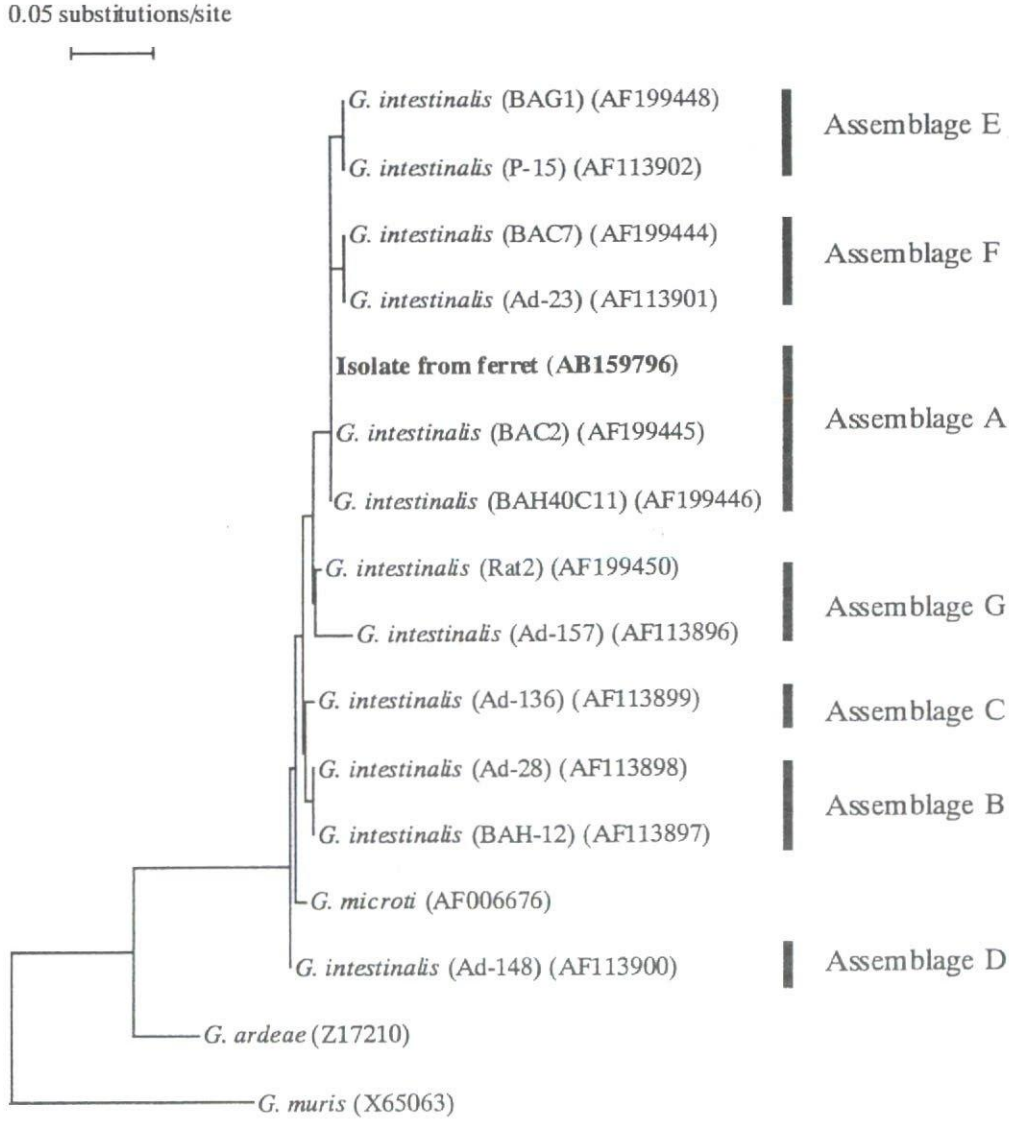

FIGURE 1. Phylogenetic relationships of the ferret isolate to other *Giardia* species and *G. intestinalis* genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of the SSUrDNA. Names of the isolates and accession numbers in GenBank are shown in parentheses.

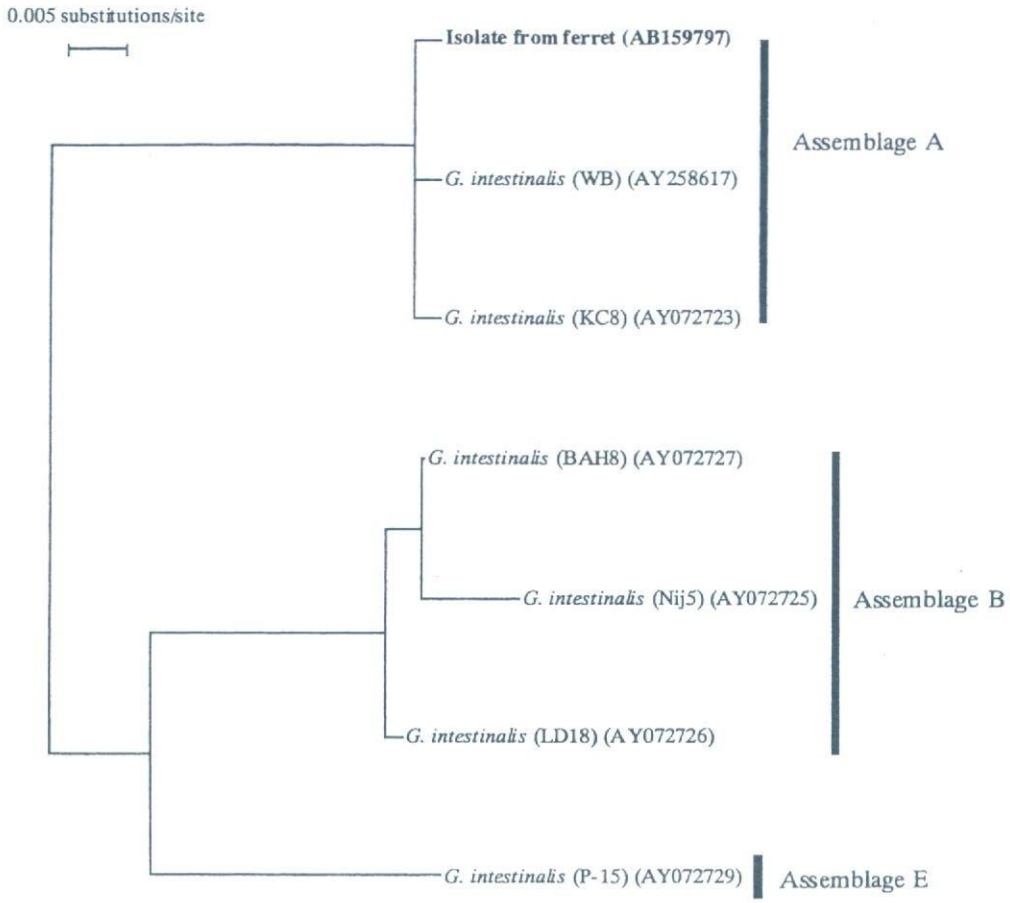

FIGURE 2. Phylogenetic relationships of the ferret isolate to other *Giardia* species and *G. intestinalis* genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of the β-giardin. Names of the isolates and accession numbers in GenBank are shown in parentheses.

and ultrastructure of their trophozoites, are recognized as valid (Adam, 2001). However, recent molecular studies have shown that *G. intestinalis* is composed of at least 7 genetically distinct, but morphologically identical, assemblages (assemblages A–G), and, moreover, most of these assemblages appear to have different host preferences, e.g., assemblages C and D in dogs, assemblage E in hoofed livestock, assemblage F in cats, and assemblage G in rats (Monis et al., 1999; Adam, 2001; Monis and Thompson, 2003). On the other hand, assemblage A consists of isolates that can be classified into 2 genetic groups (A-I and A-II) (Thompson et al., 2000). Genetic group A-I consists of a mixture of animal and human isolates. In contrast, group A-II consists entirely of human isolates. Assemblage B consists of a genetically diverse group of mainly human isolates, but some isolates from animals have been included. Therefore, it is supposed that genetic group A-I in assemblages A and B has the potential for zoonotic transmission (Thompson et al., 2000; Monis and Thompson, 2003).

At present, there has been only 1 study of *Giardia* infection in Mustelidae animals, but the genotype of the isolate remains unclear because identification was performed with only conventional microscopy (Williams et al., 1988). In Japan, the ferret is a popular pet sold in many shops, but a detailed survey of zoonotic pathogens in ferrets has not been performed (Abe and Iseki, 2003). Because *G. intestinalis* is genetically diverse and some isolates from animals appear to have zoonotic potential as mentioned above, it is likely that ferrets harbor ferret-specific or zoonotic genotypes. Therefore, it is important to analyze the isolates from ferrets genetically to elucidate the epizootiology of *Giardia* infection in animals as well as for the control of human giardiasis. In this study, we obtained an isolate from a ferret in a pet shop and compared it genetically with the multiple genotypes of *G. intestinalis* reported previously to validate the phylogenetic relationships.

A fecal sample was collected from a ferret exhibited at a pet shop in Kanazawa City, Japan. This animal showed no clinical symptoms, such as diarrhea, when the fecal sample was collected. The purification of *Giardia* cysts from the fecal sample and the extraction of DNA from cysts were performed following a method reported previously (Abe et al., 2003). *Giardia* diagnostic fragments were amplified by the polymerase chain reaction (PCR) with the following primer pairs targeting the different gene loci: RH11 and RH4 for the *Giardia* small subunit ribosomal RNA gene (SSUrDNA) (Hopkins et al., 1997), G7 and G759 for the *Giardia* β-giardin gene (β-giardin) (Cacciò et al., 2002), and GDH1 and GDH4 for the *Giardia* glutamate dehydrogenase gene (GDH) (Homan et al., 1998). The area amplified with each primer pair includes a variable region, which can be used to distinguish *Giardia* species as well as multiple genotypes of *G. intestinalis*. PCR amplification was performed under conditions reported previously (Hopkins et al., 1997; Homan et al., 1998; Cacciò et al., 2002), except that Ex *Taq* DNA polymerase, Ex *Taq* buffer, and deoxynucleoside triphosphate (TAKARA Shuzo Co. Ltd., Otsu, Japan) were used in this study. Amplification products were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide, and observed on a UV transilluminator. The PCR products were gel purified using a QIA quick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, California) on an ABI 310 automated sequencer (PE Applied Biosystems). PCR products were sequenced in both directions using each primer pair mentioned above. Sequences obtained from the ferret *Giardia* isolate were aligned with available nucleotide sequences reported previously (Baruch et al., 1996; Monis et al., 1996, 1998, 1999; Thompson et al., 2000; Cacciò et al., 2002) from other *Giardia* species and multiple genotypes of *G. intestinalis* using Clustal-X (version 1.63b). Evolutionary distance between different isolates was calculated with the Kimura 2-parameter

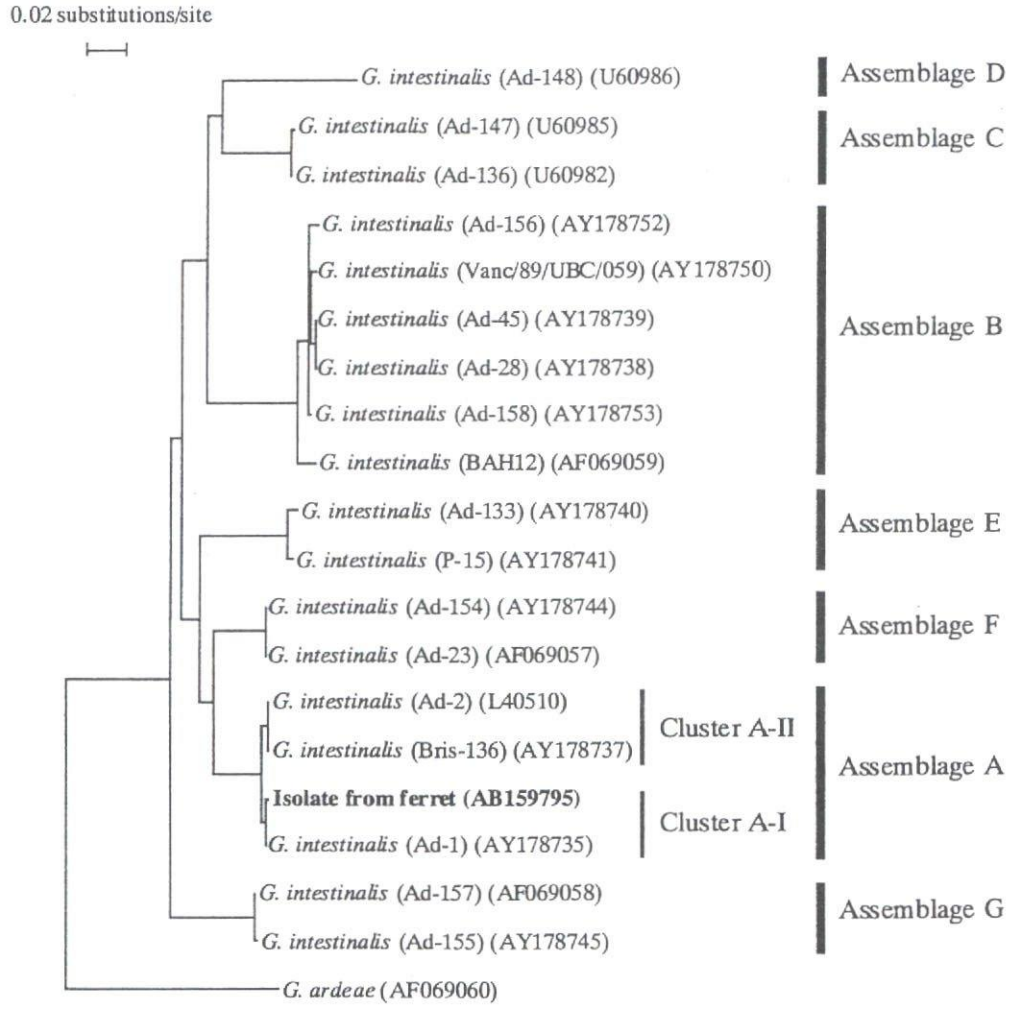

FIGURE 3. Phylogenetic relationships of the ferret isolate to other *Giardia* species and *G. intestinalis* genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of the GDH. Names of the isolates and accession numbers in GenBank are shown in parentheses.

method. Trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987) and were drawn using the NJplot program (Perrière and Gouy, 1996). The partial sequences of SSUrDNA, β-giardin, and GDH of the ferret *Giardia* isolate, obtained in this study, have been deposited in the GenBank database as AB159796, AB159797, and AB159795, respectively.

The partial SSUrDNA, β-giardin, and GDH were amplified successfully in the ferret isolate (data not shown). The partial SSUrDNA (125 bp) sequence was identical to sequences of the isolates (BAC2 and BAH40C11) found to have the assemblage A. The partial β-giardin sequence (472 bp) of the ferret isolate differed slightly from the sequences of the isolates (WB, KC8) found to have the assemblage A. There were 2 substitutions in the partial β-giardin sequence of the isolate from the ferret as compared with that of the isolate WB or KC8 (data not shown). The partial GDH sequence (592 bp) of the ferret isolate also differed slightly from the sequences of the isolates found to have the assemblage A (Ad-1, Ad-2, Bris-136). There were 1, or 3, substitutions in the partial GDH sequence as compared with that of the isolate Ad-1 or Ad-2 and Bris-136, respectively (data not shown). The close relatedness of the ferret isolate to assemblage A was also reflected in the phylogenetic analysis of β-giardin (Fig. 2) as well as SSUrDNA (Fig. 1): the ferret isolate was clustered with assemblage A. Similarly, the phylogenetic analysis of GDH sequences showed a close relatedness between the ferret isolate and assemblage A, but the ferret isolate was not clustered with the isolates Ad-2 and Bris-136 found to have group A-II but with the isolate Ad-1 found to have group A-I (Fig. 3). At present, the isolates classified into the genetic group A-I have been found in a variety of mammals, e.g., cattle, pig, horse, cat, dog, beaver, and humans, but the isolates in group A-II have been found only in humans (Adam, 2001; Monis and Thompson, 2003). Therefore, on the basis of the results of the phylogenetic analysis performed in this study and of the molecular epidemiological evidence revealed previously, we place the ferret isolate in genetic group A-I, which appears to have zoonotic potential. Although *Giardia* infection in Mustelidae had been confirmed already in a black-footed ferret, *Mustela nigripes*, by light microscopy in 1988 (Williams et al., 1988), since then there have been no reports regarding *Giardia* infection in weasels. Therefore, our study is the first molecular analysis of an isolate from weasels. Epizootiological surveys of zoonotic pathogens in animals reared in pet shops or by breeders have been overlooked, and thus, periodical examinations of pets are needed to prevent infections with zoonotic pathogens.

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SHORT COMMUNICATION

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Identification of *Cryptosporidium* isolates from cockatiels by direct sequencing of the PCR-amplified small subunit ribosomal RNA gene

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Abstract *Cryptosporidium* is a significant pathogen in humans and animals. Cases of infection by *C. meleagridis* or *C. baileyi* with zoonotic potential have also been reported in domestic birds; and recent studies indicate the presence of new host-adapted species or genotype in birds. Therefore, accurately identifying isolates is important for understanding the epizootiology of *Cryptosporidium* infection in birds and for the control of human cryptosporidiosis. *Cryptosporidium* has been detected in cockatiels, but the species or genotype of isolates remains unclear because identification was performed using conventional microscopy. We report herein the species or genotype of isolates from two cockatiels distinguished by a PCR-based diagnostic method. The isolates were found to be *C. meleagridis* and *C. baileyi*, respectively. This study documents the first discovery of *C. meleagridis* and *C. baileyi* in cockatiels and suggests that pet birds may play an important role in the epidemiology of cryptosporidiosis.

Cryptosporidium is a protozoan parasite that is ubiquitous in its geographic distribution and range of vertebrate hosts. In domestic birds, especially in chickens and turkeys, *C. meleagridis* and *C. baileyi* are recognized as significant pathogens, primarily as a cause of diarrheal and respiratory illness, respectively (Ditrich et al. 1991; Sréter and Varga 2000). Both species are also known to be zoonotic (Xiao et al. 2000). Although *Cryptosporidium* organisms have been found in popular pet birds belonging to the orders Passeriformes and Psittaciformes, in most cases of infection reported to date, identification has been based on only the conventional microscopy of intestinal tissue or fecal samples (O'Donoghue 1995). Recently, isolates which are indistinguishable morphologically but quite different genetically from other *Cryptosporidium* species or genotypes, have been found in finches, a black duck and a goose and are proposed as new species (*C. blagburni* from finches, *Cryptosporidium* sp. from the black duck) and genotypes (*Cryptosporidium* goose genotype from the goose; Morgan et al. 2001; Xiao et al. 2002). Meanwhile, it was demonstrated using a PCR-based method that an aviary-bred parrot was infected with *C. meleagridis* (Morgan et al. 2000). Therefore, it is important to accurately identify avian isolates in order to elucidate the epizootiology of *Cryptosporidium* infection in birds and for the control of human cryptosporidiosis.

In Japan, the cockatiel is a popular companion sold in many pet shops, but no surveys of zoonotic pathogens in cockatiels has been performed. In addition, there have been only two studies of *Cryptosporidium* infection in cockatiels, neither of which accurately identified the isolates obtained (Goodwin and Krabill 1989; Lindsay et al. 1990). In the present study, we obtained two isolates from cockatiels in a pet shop and identified the species or genotype by directly sequencing the PCR-amplified small subunit ribosomal RNA gene.

Fecal samples were collected from two cockatiels exhibited at a pet shop in Kanazawa City, Japan. Neither bird showed clinical symptoms when the fecal samples were collected. The concentration of *Cryptosporidium* oocysts from fecal samples and the extraction of DNA from oocysts were performed as reported by Abe et al. (2002a, 2002b). The *Cryptosporidium* diagnostic fragment was amplified by nested-PCR targeting the small subunit ribosomal RNA gene of *Cryptosporidium* reported by Xiao et al. (1999). The area amplified includes a variable region that can be used to distinguish among *Cryptosporidium* species and genotypes by PCR–RFLP or phylogenetic analysis (Xiao et al. 1999).

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However, we recently showed that PCR–RFLP of this region could not be used to distinguish among certain *Cryptosporidium* species and genotypes because of a similarity in RFLP profiles (Abe et al. 2002c). Therefore, in the present study, we identified the isolates from cockatiels by phylogenetic analysis, using data obtained from the direct sequencing of PCR products.

For the primary PCR step, a product approximately 1,300 bp long was amplified in a volume of 50 µl containing 1× PCR buffer, 2 m *M* $MgCl_2$, 250 µ*M* each dNTP, 0.5 µ*M* each primary primer reported by Xiao et al. (1999), 1.25 units of Ex *Taq* DNA polymerase (Takara Shuzo Co., Otsu, Japan) and 5 µl of the DNA sample. We used the PCR buffer and dNTP mixture supplied with Ex *Taq* DNA polymerase. Reactions were performed on a GeneAmp PCR System 9700 thermocycler (Perkin–Elmer Co., Foster City, Calif.). Samples were denatured at 94 °C for 3 min and then subjected to 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. For the secondary PCR step, a product approximately 830 bp long was amplified using 2 µl of the primary PCR product and a secondary primer pair reported by Xiao et al. (1999). The PCR mixture and cycling conditions were identical to those used for the primary PCR step. The DNA of *C. parvum* strain HNJ-1 originating from a patient was used as a positive control for the PCR. This strain had the cattle genotype (Abe et al. 2002a). Products of the amplification were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator.

The PCR products were gel-purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced using an ABI Prism BigDye terminator cycle sequencing FS ready reaction kit (Perkin–Elmer Co.) on an automated sequencer (ABI Prism model 310; Perkin–Elmer Co.). They were sequenced in both directions, using a secondary primer pair. Sequences obtained from the cockatiel isolates were aligned with nucleotide sequences obtained from other *Cryptosporidium* species and genotypes (Cai et al. 1992; Johnson et al. 1995; Kimbell et al. 1999; Morgan et al. 1999, 2001; Xiao et al. 1999, 2002; Akiyoshi et al. 2003; Satou et al. 2003), using Clustal-X ver. 1.63b. The evolutionary distance between different isolates was calculated with the Kimura two-parameter method. Trees were constructed using the neighbor-joining algorithm (Saitou and Nei 1987). Branch reliability was assessed using bootstrap analyses (1,000 replicates) and the phylograms were drawn using the NJplot program (Perrière and Gouy 1996).

Agarose gel visualization of nested-PCR products revealed similar-sized diagnostic fragments (approximately 830 bp) for HNJ-1 and isolate A from one cockatiel (Fig. 1, lanes 1, 2), but the fragment in isolate B from the other cockatiel was slightly smaller (Fig. 1, lane 3). The DNA sequences of the diagnostic fragments of isolates A and B were 833 bases and 826 bases long, respectively, and clearly clustered with the isolates

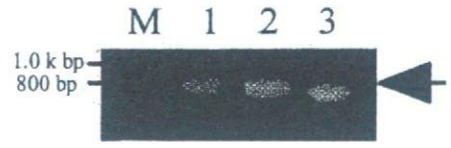

Fig. 1 Detection of *Cryptosporidium*-specific fragments (approximately 830 bp long) by nested-PCR. *Lane M* Molecular marker (100-bp ladder), *lane 1 C. parvum* strain HNJ-1, *lanes 2, 3* isolates A and B from cockatiels. The *arrow* shows the *Cryptosporidium* diagnostic fragment amplified by nested-PCR

known to have *C. meleagridis* and *C. baileyi*, respectively (Fig. 2). Therefore, the isolates from cockatiels examined in the present study were identified as *C. meleagridis* and *C. baileyi*, respectively.

The first case of *Cryptosporidium* infection in cockatiels was reported in 1989 in the United States (Goodwin and Krabill 1989). The next year, a second case was reported in the same country (Lindsay et al. 1990). Since then, however, there have been no reports of *Cryptosporidium* infection in cockatiels. In addition, neither the species nor genotype of the isolates described in those studies was identified, though *C. baileyi* was suspected to have caused the second case, based on the size of oocysts. Therefore, it remains unclear what species or genotypes of *Cryptosporidium* are harbored in cockatiels. The present study is the third report of *Cryptosporidium* infection in cockatiels and it documents the first positive identification of *C. baileyi* and *C. meleagridis* in cockatiels.

The pathogenicity of *C. meleagridis* and *C. baileyi* in cockatiels is still unclear, but in both previous cases the infection was acute (Goodwin and Krabill 1989; Lindsay et al. 1990). In the first case, Goodwin et al. (1989) diagnosed the cause of death as inhalation pneumonia. In the second case, it was unclear whether the cause of death was related to *Cryptosporidium*, because of concurrent infections with other pathogens (Lindsay et al. 1990). In the present study, neither of the cockatiels infected with *C. meleagridis* or *C. baileyi* showed clinical symptoms. Therefore, further study is required to fully understand the veterinary significance of these two species in cockatiels.

According to recent molecular epidemiological studies, *C. meleagridis* has been found in both immunocompetent and immunocompromised patients (Xiao et al. 2000; Yagita et al. 2001). In addition, there has been a report of the excretion of *C. baileyi* oocysts in an immunocompromised patient and endogenous stages have been found in organs taken at autopsy (Ditrich et al. 1991). Therefore, both *Cryptosporidium* species are recognized as zoonotic. *Cryptosporidium* organisms have been found in pet birds, such as the cockatiel, canary, finch, lovebird, parrot and budgerigar (O'Donoghue 1995) and cryptosporidial infections in these birds may be more common than previously thought. Zoonotic transmission may occur from these avian sources; and therefore periodic examinations of pet birds are necessary to prevent infections from zoonotic pathogens.

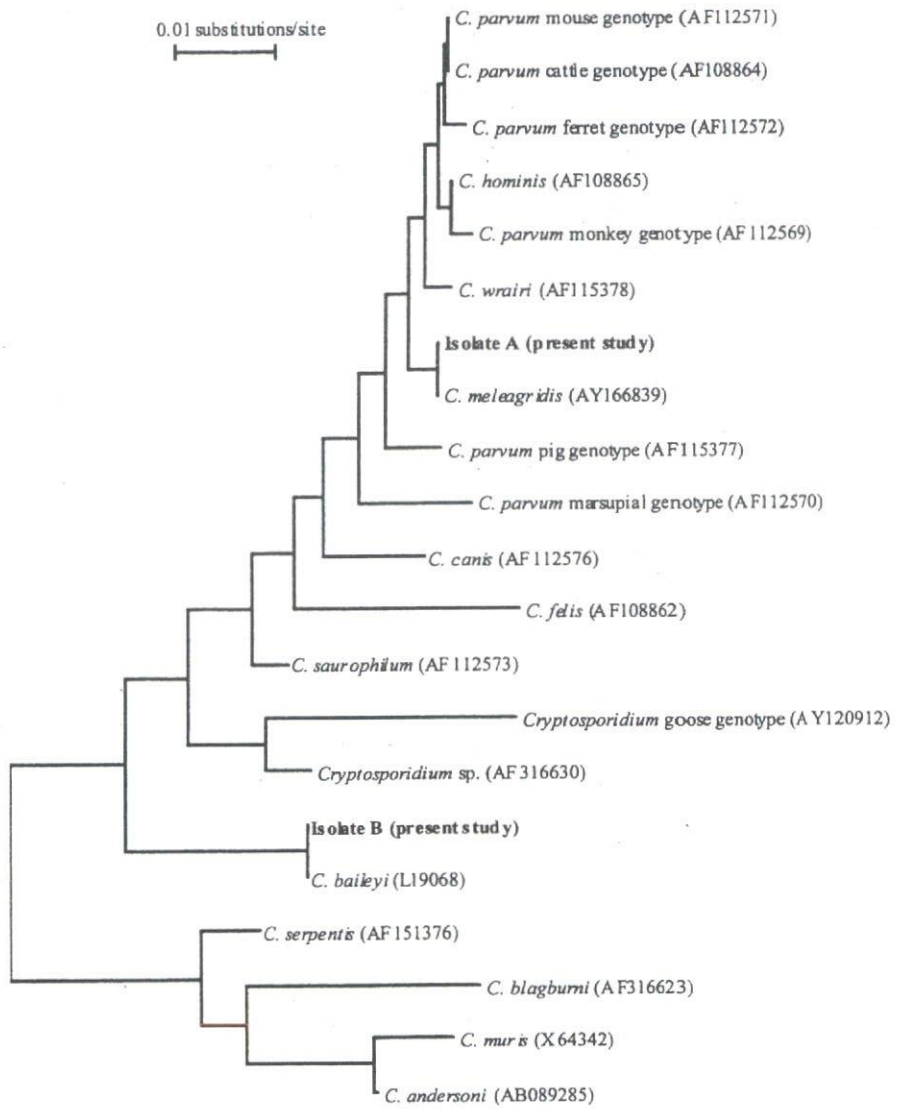

Fig. 2 Phylogram of the two isolates (A, B) from cockatiels and other *Cryptosporidium* species and genotypes, as inferred by neighbor-joining analysis of nucleotide sequences of the small subunit ribosomal RNA gene. GenBank accession numbers of the *Cryptosporidium* organisms represented are shown *in parentheses*

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First record of *Cryptosporidium* infection in a raccoon dog (*Nyctereutes procyonoides viverrinus*)

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Abstract

Cryptosporidium species have been found in more than 150 species of mammals, but there has been no report in raccoon dogs. Here we found the *Cryptosporidium* organism in a raccoon dog, *Nyctereutes procyonoides viverrinus*, and identified this isolate using PCR-based diagnostic methods. *Cryptosporidium* diagnostic fragments of the 18S ribosomal RNA, *Cryptosporidium* oocyst wall protein and 70-kDa heat shock protein genes were amplified from the isolate and sequenced to reveal the phylogenetic relationships between it and other *Cryptosporidium* species or genotypes reported previously. The results showed that the raccoon dog isolate represented the *C. parvum* cattle genotype which could be a causative agent in human cryptosporidiosis.

Keywords: *Cryptosporidium*; Genotype; Japan; Raccoon dog

Cryptosporidium parvum is an apicomplexan parasite found in a variety of mammals including humans. In immunocompetent hosts, the infection is typically acute and self-limiting, whereas in immunocompromised individuals, cryptosporidiosis is often a chronic disease (Guyot et al., 2002). Although outbreaks of cryptosporidiosis are associated with indirect

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transmission via contaminated food or public water supplies, the source of infection is thought to be either infected animal feces or human sewage.

Recent molecular studies show that *C. parvum* is composed of genetically distinct but morphologically identical genotypes (Morgan et al., 1997, 1999b; Xiao et al., 2000b). Among these genotypes, the human (recently proposed as a new species, *C. hominis* (Morgan-Ryan et al., 2002)) and cattle genotypes are found to be the main causative agents in human cryptosporidiosis (Guyot et al., 2001), but both genotypes have clearly distinct features of transmission to heterogeneous hosts. As well as affecting humans, the human genotype has showed infectivity in a piglet (Widmer et al., 2000), and been found in a single nonhuman primate (Spano et al., 1998) and a dugong (Morgan et al., 2000b), while the cattle genotype has been found to easily infect laboratory animals such as rodents and in a variety of ruminants as well as humans (Morgan et al., 1999b, 2000a; Xiao et al., 2002). Therefore, the latter is commonly recognized as a zoonotic genotype of *C. parvum*.

A variety of wild animals have been reported to be infected with *C. parvum* and *C. parvum*-like organisms (Fayer et al., 2000). However, few of the isolates have been analyzed genetically. It was confirmed that isolates from cattle could infect a variety of animals, but the isolates used were not genotyped (Fayer and Ungar, 1986). Therefore, it is likely that wild animals harbor the *C. parvum* cattle genotype. In October 2000, an injured wild raccoon dog was brought to Osaka Municipal Tennoji Zoological Gardens and treated at a hospital there. While in quarantine, a fecal sample was collected from this animal and examined by light microscopy and immunofluorescence antibody tests using two commercially available kits (Hydrofluor-Combo, Ensys, NC; MELIFLUOR, Meridian, Ohio) to detect *Cryptosporidium* oocysts. Consequently, oocysts morphologically similar to those of *C. parvum* were found in the sample. Since *Cryptosporidium* parasites have not been found previously in raccoon dogs, we speculated that this isolate was zoonotic or a new genotype. Thus, we compared it genetically with other *Cryptosporidium* species or genotypes reported previously, to confirm the phylogenetic relationships.

The purification of *Cryptosporidium* oocysts from the fecal sample and the extraction of DNA from the oocysts were performed as reported (Abe et al., 2002c). *Cryptosporidium* diagnostic fragments were amplified by PCR with the following primer pairs targeting the different gene loci: 18SiF and 18SiR for the *Cryptosporidium* 18S ribosomal RNA gene (18SrDNA) (Morgan et al., 1997), chsp1 and chsp4 for the *Cryptosporidium* 70-kDa heat shock protein gene (HSP70) (Gobet and Toze, 2001), and cry15 and cry9 for the *Cryptosporidium* oocyst wall protein gene (COWP) (Spano et al., 1997). The area amplified with each primer pair includes a variable region which can be used to distinguish among *Cryptosporidium* species as well as genotypes (Xiao et al., 2000a; Gobet and Toze, 2001; Abe et al., 2002b). PCR amplification was performed under conditions reported previously (Abe et al., 2002a; Abe and Iseki, 2003). The DNA of *C. parvum* strain HNJ-1 originating from a patient was used as a positive control for the PCR. This strain has the cattle genotype of *C. parvum* (Abe et al., 2002a). Amplification products were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide and visualized on an UV transilluminator. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, USA) on an automated sequencer (ABI PRISM 310 model; Perkin-Elmer, USA). They were then sequenced in both directions

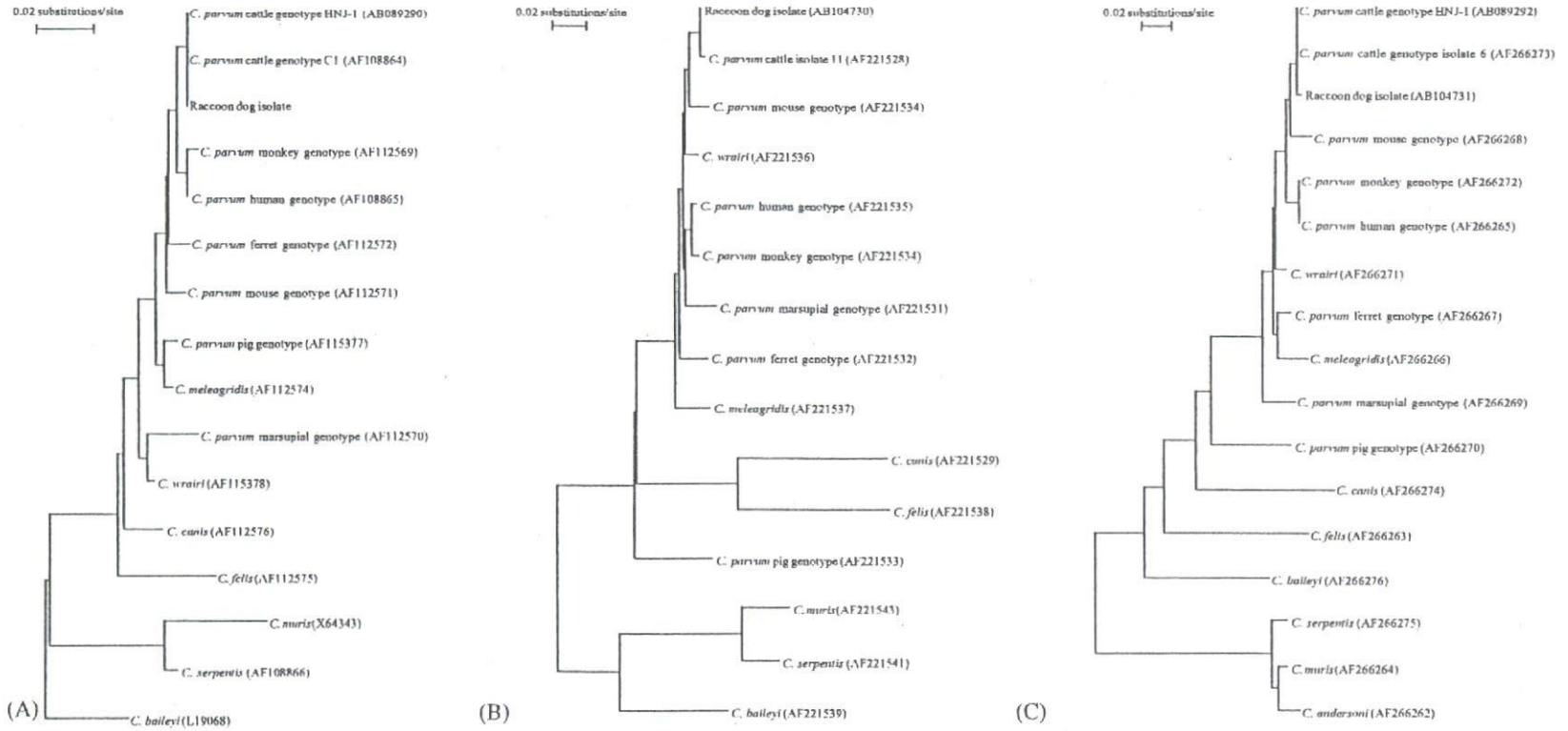

Fig. 1. Phylogenetic relationship of the raccoon dog isolate to other *Cryptosporidium* species or genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of 18SrDNA (A), HSP70 (B) and COWP (C). The GenBank accession numbers of each *Cryptosporidium* species or genotype are shown in parentheses.

using each primer pair mentioned above. Sequences obtained from the raccoon dog isolate were aligned using Clustal-X (version 1.63b; December 1997). The evolutionary distance between different isolates was calculated using the Kimura 2-parameter method, and phylogenetic trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987).

The partial 18SrDNA, HSP70 and COWP sequences were successfully amplified in the raccoon dog isolate (data not shown). The partial 18SrDNA sequence was 295 bases long and completely identical to sequences of isolates (C1, HNJ-1) found to have the cattle genotype (Morgan et al., 1999a; Satoh et al., 2003). The sequences of the partial HSP70 and COWP were 587 and 553 bases long, respectively, but both differed slightly from those of isolates (HNJ-1, isolates 6 and 11) found to have the cattle genotype (Sulaiman et al., 2000; Xiao et al., 2000a; Satoh et al., 2003). There were two substitutions in the partial HSP70 sequence of the isolate from the raccoon dog as compared with that of isolate 11 (data not shown). The partial COWP sequences of HNJ-1 and isolate 6 were identical, but differed slightly for the sequence of the isolate from the raccoon dog (data not shown). Thus, the partial sequences of HSP70 and COWP from the raccoon dog isolate, obtained in the present study, have been deposited in GenBank under accession numbers AB104730 and AB104731, respectively. A phylogenetic tree was constructed based on the neighbor-joining analysis of nucleotide sequences of 18SrDNA, HSP70 and COWP to confirm the phylogenetic relationships of the isolate from the raccoon dog among *Cryptosporidium* species or genotypes (Fig. 1). As shown in Fig. 1, the close relatedness of the raccoon dog isolate to the *C. parvum* cattle genotype was also reflected in the phylogenetic analyses of HSP70 (Fig. 1B) and COWP (Fig. 1C) as well as 18SrDNA (Fig. 1A). By these analyses, the raccoon dog isolate was clustered with the isolates found to have the cattle genotype (Fig. 1B and C). On the basis of the results obtained from phylogenetic analyses, we identified this isolate as having the *C. parvum* cattle genotype.

Although *Cryptosporidium* and *Cryptosporidium*-like organisms have been found in more than 150 species of animals (Fayer et al., 2000), there has been no report of *Cryptosporidium* infection in raccoon dogs. Since natural infections with the *C. parvum* cattle genotype other than in ruminants and humans have been confirmed in only an immunosuppressed dog (Fayer et al., 2001), the distribution of this genotype in wild animals has been unclear. Therefore, our study is the first record of *Cryptosporidium* infection in a raccoon dog, and also indicates the possibility of infections with the *C. parvum* cattle genotype in many more wild animals. The raccoon dog is a common species in Japan found in both urban and rural environments. For this reason, it is speculated that cross-transmission could occur between humans and raccoon dogs. Further molecular analyses of these isolates are required to test this hypothesis.

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