

DETECTION OF NEW *ENTEROCYTOZOOON* GENOTYPES IN FAECAL SAMPLES OF FARM DOGS AND A CAT

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Summary :

The microsporidian species *Enterocytozoon bienewsi* had emerged as opportunistic pathogen in AIDS patients causing chronic diarrhoea and was found with high prevalences in faeces of asymptomatic pigs. Analysis of the ribosomal RNA gene internal transcribed spacer (rDNA ITS) had revealed that nine distinct but closely related genotypes occur in humans and in swine. Using primers that were designed to be specific for *E. bienewsi*, we obtained amplicons from the faecal samples of one from twelve cats and from three out of 36 farm dogs. Sequence analysis of the rDNA ITS, which is part of the diagnostic PCR product, revealed that the isolate from the cat is very closely related to the *E. bienewsi* genotypes of human or swine origin. The corresponding sequence of all three dog-derived isolates were identical among each other and had a sequence similarity to known sequences of only 47.6-48.2 %. In addition, part of the small subunit rRNA gene was amplified and sequenced from one dog-derived isolate revealing a similarity to known sequences of human-derived *E. bienewsi* of 96-98 %. *Enterocytozoon*-like spores could be detected by light microscopy in one canine sample. Together with recent reports of detection of *Enterocytozoon* in environmental samples, our findings suggest that microsporidia of the genus *Enterocytozoon* seem to be ubiquitous and consist of many genotypes in various naturally infected animal species.

KEY WORDS : *Enterocytozoon bienewsi*, dog, cat, genotypes, zoonosis, HIV.

Résumé :

PRÉSENCE, DANS DES FERMES DE NOUVEAUX GÉNOTYPES D'*ENTEROCYTOZOOON* DANS LES MATIÈRES FÉCALES DE CHIENS ET D'UN CHAT. *Enterocytozoon bienewsi* s'est révélé être une microsporidie pathogène opportuniste chez les malades atteints de SIDA et détermine des diarrhées chroniques. Elle a une haute prévalence chez les porcs, même asymptomatiques. L'analyse de la séquence ITS du gène codant l'ARN ribosomique (rDNA ITS) a montré l'existence de neuf génotypes proches mais distincts chez l'homme et le porc. En utilisant les amorces qui sont réputées spécifiques de *E. bienewsi*, il a été obtenu des amplicons dans les matières fécales d'un chat sur 12 et de trois chiens de ferme sur 36. L'analyse de la séquence ITS du rDNA avec laquelle a été exécutée la PCR montre que la souche isolée du chat est très proche des génotypes de *E. bienewsi* d'origine humaine ou porcine. La séquence correspondante, identique chez les trois isolats provenant des chiens, n'est similaire qu'à 47,6-48,2 % des séquences connues. En outre, une partie de la petite sous-unité du gène rDNA ayant été amplifiée et séquencée à partir d'un des isolats canins a révélé une similitude de 96-98 % avec les séquences connues chez *E. bienewsi* d'origine humaine. Des spores du type *Enterocytozoon* ont été détectées en microscopie optique dans les matières fécales d'un chien. En accord avec les travaux récents montrant la présence d'*Enterocytozoon* dans l'environnement, nos données suggèrent que le genre *Enterocytozoon* semble ubiquiste et comprend de nombreux génotypes chez de nombreux animaux spontanément infectés.

MOTS CLÉS : *Enterocytozoon bienewsi*, chien, chat, génotype, zoonose, VIH.

Microsporidiosis had emerged as an AIDS-related opportunistic infection. The species *Enterocytozoon bienewsi* which was first described in 1985 as a spore-forming organism that infected the enterocytes of a patient with AIDS (Desportes *et al.*, 1985), seems to be the most common microsporidian causing chronic diarrhoea with high prevalences in AIDS patients, but also affects patients with therapeutic immunodeficiencies (Desportes-Livage, 1998; Rabodonirina *et al.*, 1996). Analysis of the rDNA internal transcribed spacer (ITS) of *E. bienewsi*

revealed that at least five different genotypes occur in humans (Rinder *et al.*, 1997; Liguory *et al.*, 1998). The detection of *E. bienewsi* in samples of four pigs (Deplazes *et al.*, 1996) raised the question of a zoonotic potential of this parasite. In a subsequent study (Breitenmoser *et al.*, 1999), the prevalence of *E. bienewsi* was found to be 35 % among 109 randomly selected asymptomatic pigs from 22 farms from Switzerland. Four genotypes of *E. bienewsi* from pig origin could be distinguished as assessed by sequence variability in the rDNA ITS which slightly differed (identity 96.3-98.9 %) from four human-derived *E. bienewsi* genotypes (Rinder *et al.*, 1997). Three of those human genotypes could also be detected in specimens of 13 AIDS patients from Switzerland. Hence, *E. bienewsi* isolates from humans and pigs in Switzerland seem to be epidemiologically unrelated but the zoonotic potential of the swine-derived genotypes remains to be elu-

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cidated. So far, only one other natural infection with *E. bienersi* was documented in animals, namely in simian-immunodeficiency-virus (SIV)-inoculated macaques with hepatobiliary disease. These animals were kept in a biolevel 3 containment facility and it was suggested that the disease either resulted from the reactivation of an existing infection or from the transmission of this microsporidium by fomites (Mansfield *et al.*, 1997). The ITS sequence from this simian *E. bienersi* (Mansfield *et al.*, GenBank accession number AF023245) was 100 % identical to the human-derived *E. bienersi* genotype D (GenBank accession no. AF101200).

E. bienersi was recently also identified in environmental surface water in France and the USA (Sparfel *et al.*, 1997; Dowd *et al.*, 1998) by sequence analysis of PCR amplicons suggesting that this parasite is ubiquitous and might have natural hosts other than swine. We here report on the identification and genetic characterisation of *Enterocytozoon* from two other host species, dog and cat.

MATERIALS AND METHODS

Thirty-six faecal samples from free roaming dogs and 12 from cats from different farms from the Western part of Switzerland were available. Faecal samples were stored at 4°C until microscopic examination, and an aliquot of about 1 g was stored at -20°C for PCR. For the microscopical detection of microsporidial spores, slides were prepared from 10 µl of unconcentrated stool and stained with the chromotrope stain (Weber *et al.*, 1992). Slides were scanned at a magnification of 1000× with oil immersion for 15 minutes by an experienced microscopist.

For DNA isolation with the alkaline lysis method described by Katzwinkel-Wladarsch *et al.* (1996), approximately 0.1 g of unconcentrated stool samples were used. Screening of the samples was done with the PCR according to Katzwinkel-Wladarsch *et al.* (1996) using the primer pair MSP3 and MSP4B as described (Breitenmoser *et al.*, 1999). This system includes the use of a size-modified control target to detect inhibition of the amplification reactions as well as the use of a carry-over prevention system (uracil-N-glycosylase). These primers amplify a 508 bp fragment of *E. bienersi* containing 122 bp of the 3' end of the SSU rRNA gene, 243 bp of the internal transcribed spacer (ITS) and 143 bp of the 5' region of the LSU rRNA gene. In addition the *E. bienersi*-specific primers Ebief and Ebier were used amplifying a 607 bp fragment of the small subunit rDNA (da Silva *et al.*, 1996). All positive samples were independently examined twice (DNA extraction, amplification and sequencing). DNA sequencing was

performed by automated means (ABI PRISM System, Perkin Elmer) by a private company (Microsynth, Balgach, Switzerland). All sequence analyses were performed with default settings using the GCG software package (Version 8, Genetics Computer Group, Madison, WI) running on Silicon Graphics, Challenge.

RESULTS

From the total 48 faecal samples of 36 farm dogs and 12 cats, three from dogs (prevalence 8.3 %) and one from a cat (prevalence 9 %) were identified as being *Enterocytozoon*-positive by the presence of PCR products of the expected size of 508 bp. Sequence analyses of these products showed that a 106 bp long SSU rDNA fragment at the 5' end of all PCR products was 100 % identical with the corresponding published sequences of *E. bienersi* isolates from humans and from pigs. Sequence analysis of the rDNA ITS revealed that the genotype from the cat (GenBank accession no. AF118144) has not been described before and is most closely related to the human-derived genotype D differing at two positions among the total 243 bp (Fig. 1). All ITS sequences from *Enterocytozoon* from the three dogs were also 243 bp in length and identical among each other (GenBank accession no. AF059610) but exhibited only 47.6-48.2 % similarity to the sequences from human- and pig-derived *E. bienersi* genotypes. With DNA from the

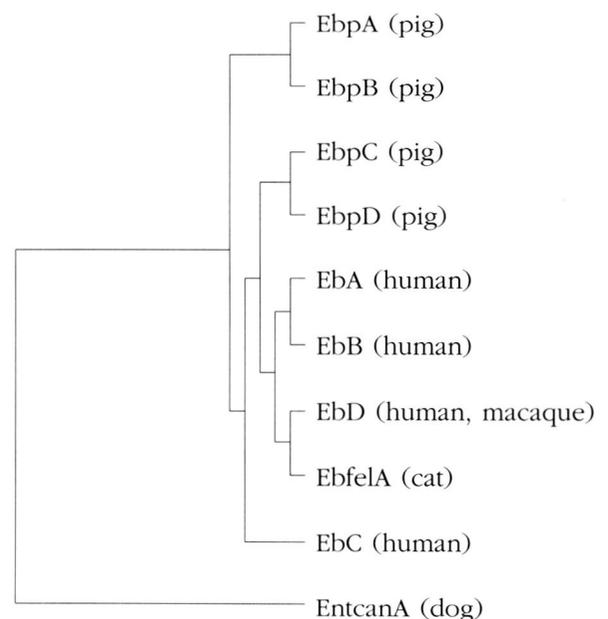


Fig. 1. – Dendrogram of rDNA ITS sequences of *E. bienersi* (Eb) from humans and macaques (A-D) (Rinder *et al.*, 1997; Mansfield *et al.*, 1997), swine (pA-pD) (Breitenmoser *et al.*, 1999), cat (felA) and from *Enterocytozoon* from dog (EntcanA).

faecal sample of dog no. 62 an additional 538 bp long fragment of the SSU rDNA was amplified and sequenced (GenBank accession no. AF119100) revealing a similarity to known *E. bienewsi* sequences of 96 and 98 %, respectively.

Single spores characteristic for *Enterocytozoon* could be detected by light microscopy using the chromotrope stain in the faecal sample from one dog (no. 62) only.

DISCUSSION

By using primers which were designed to be specific for *E. bienewsi*, we identified dog and cat as two new natural animal host species. All microsporidial isolates were identified as belonging to the genus *Enterocytozoon* by sequence identity of 106 bp of the SSU rRNA gene. Comparison of the same part of this gene with that from *Nucleospora* (formerly *Enterocytozoon*) *salmonis*, the most closely related known species (Docker *et al.*, 1997), showed only 86.9 % identity. Such a variability of around 15 % in the SSU rDNA sequence corresponds to results obtained when comparing the sequences from closely related microsporidial species like *Encephalitozoon cuniculi* and *E. hellem* (Mathis *et al.*, 1997). Genetic analyses of the rDNA ITS revealed that the isolate from the cat represents a new *E. bienewsi* genotype which clusters very closely together with the four genotypes from swine and three of the four genotypes found in humans (Breitenmoser *et al.*, 1999). The three isolates from dogs, however, were distinctly different. Their ITS sequence has an identity of less than 50 % as compared with the known sequences of human- and pig-derived genotypes (Rinder *et al.*, 1997; da Silva *et al.*, 1998, Genbank accession no. AF024657; Deplazes *et al.*, 1996, Breitenmoser *et al.*, 1999) and would also represent a new RFLP pattern when applying the system described by Liguory *et al.* (1998) which allowed to discriminate four human-derived *E. bienewsi* genotypes. The analysis of an additional 538 bp from the SSU rDNA of the dog-derived isolate revealed a variability to sequences of human-derived *E. bienewsi* of 2 % (Hartskeerl *et al.*, 1993; GenBank accession no L16868; Da Silva *et al.*, 1996; GenBank accession no AF024657) and 4% (Zhu *et al.*, 1993; GenBank accession no L07123). These data indicate that a considerable genetic variation within the genus *Enterocytozoon* exists in nature. A comparable sequence divergence among the rDNA ITS1 has previously been reported in *Cryptosporidium* with substantial length differences ranging from 363-618 bp in isolates from different hosts (Morgan *et al.*, 1999). To further clarify the taxonomic status of this dog-derived *Enterocytozoon* genotype, a multilocus genotypic analysis is needed.

Most interestingly, exactly the same variability of 2 % to the above mentioned known human-derived *E. bienewsi* SSU rDNA sequence (Hartskeerl *et al.*, 1993) was found by Sparfel *et al.* (1997) and by Dowd *et al.* (1998) for the full length SSU rRNA gene or part of this gene, respectively, of *Enterocytozoon* that they had identified from surface water in France (river Seine) and the USA, but unfortunately, these sequences are not available for comparative analysis.

The infection of dogs with *Enterocytozoon* is reminiscent to the situation in swine (Breitenmoser *et al.*, 1999): the prevalence is rather high (although only a relatively small number of dogs was examined), excretion of spores is low (as spores could only be detected in one dog-derived sample by light microscopy) and the infection seems to be asymptomatic (as deduced from the consistence of the faecal material). Similar results were found after inoculation with human-derived *E. bienewsi* in that only chronic asymptomatic infections were observed in experimental animal models (heavily immunosuppressed rats, rabbits, or piglets, Accoeberry *et al.*, 1997a, 1997b; Kondova *et al.*, 1998; SIV-infected rhesus monkeys; Tzipori *et al.*, 1997).

Our molecular and microscopical findings of *Enterocytozoon* in single faecal samples are no definitive proofs for a true intestinal infection of dogs and cats leaving the possibility of a simple transit through the gut of spores originating from water or food. However, this seems unlikely as the *Enterocytozoon* genotypes from dogs and the cat from the same area were distinctly different from each other.

All *Enterocytozoon* genotypes from naturally infected animals identified so far (swine [Breitenmoser *et al.*, 1999]; dog, cat [this study]) are different from the *E. bienewsi* genotypes identified in AIDS patients, indicating that other sources are responsible for the human infection. Hutin *et al.* (1998) identified three risk factors for human intestinal microsporidiosis caused by *E. bienewsi* or *Encephalitozoon intestinalis*: CD4 cell counts < 200/mm³, male homosexuality and swimming in pools. Neither contact with pet animals, including cats and dogs, nor exposure to different kind of food or consumption of tap water were associated with disease supporting the hypothesis that intestinal microsporidiosis is transmitted through the faecal-oral route. Whereas only single case reports are available on microsporidiosis in cats (Van Rensburg & Du Plessis, 1971; Buyukmihci *et al.*, 1977), microsporidial infections in dogs due to *Encephalitozoon cuniculi* causing encephalitis and nephritis are frequently observed in the USA and South Africa (Canning & Lom, 1986; Gevrey, 1993). Our finding of *Enterocytozoon* represents the second detection of intestinal microsporidia in dogs after the report of Bornay-Llinares *et al.* (1998) of canine infections with *Encephalitozoon intestinalis*.

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Note: Nucleotide sequence data reported in this paper are available in the GenBank data base under accession numbers AF118144 (rDNA ITS from cat-derived *E. bienersi*), AF059610 and AF119100 (rDNA ITS and partial SSU rDNA sequence, respectively, from dog-derived *Enterocytozoon sp.*)

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