whether biological differences are related to the species of the parasite or to the host or both. Much more research, particularly at the molecular level, must be done on *Uncinaria* spp. in pinnipeds before more conclusive determinations can be made regarding the taxonomy of these hookworms.

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Detection of the *Cryptosporidium parvum* "Human" Genotype in a Dugong (*Dugong dugon*)

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ABSTRACT: The *Cryptosporidium* "human" genotype was identified in a paraffin-embedded tissue section from a dugong (*Dugong dugon*) by 2 independent laboratories. DNA sequencing and polymerase chain reaction/restriction fragment length polymorphism analysis of the 18S ribosomal RNA gene and the acetyl CoA synthethase gene clearly identified the genotype as that of the *Cryptosporidium* variant that infects humans. This is the first report of the human *Cryptosporidium* genotype in a nonprimate host.

Species of *Cryptosporidium* are common parasites of the alimentary and respiratory tracts of vertebrates. Those species affecting mammals are often associated with severe diarrhea, which can be prolonged and life threatening in the very young and immunologically impaired (O'Donoghue, 1995; Fayer et al., 1997).

Direct fecal–oral transmission is a common route of infection, but in recent years, cryptosporidiosis has emerged as an important waterborne disease, resulting in numerous outbreaks of diarrhea throughout the world (O'Donoghue, 1995). As a consequence, increasing attention has focused on the need for accurate surveillance of water supplies destined for human consumption and reliable methods to determine the source of contamination in disease outbreaks. The difficulties surrounding source determination have been compounded by recent research that has shown that the most important species with respect to both public health and veterinary medicine, *Cryptosporidium parvum,* is not a uniform species but rather consists of many genotypes or strains, some of which may represent different species (Morgan, Monis, et al., 1999; Morgan, Xiao, et al., 1999; Xiao et al., 1999). Although in most cases these genotypic variants are not morphologically distinct, they do exhibit differences in host specificity. Some variants appear to be adapted to a particular host species, whereas others are capable of infecting different species and have zoonotic potential. From a public health perspective, it is important to be able to differentiate between genotypes of *Cryptosporidium* to accurately evaluate risk factors and sources of infection, particularly during disease outbreaks.

```
Dugong isolate      ACTA---TTTTTTTTTAGTAT
Monkey genotype     ACTA---TTTTTTTTTAGTAT
Human genotype120   ACTA-TTTTTTTTTTTAGTAT
Human genotype122   ACTA--TTTTTTTTTTAGTAT
Human genotype94    ACTA------TTTTTTAGTAT
Human genotype181   ACTA----TTTTTTTTAGTAT
Bovine genotype     ACTA----TATATTTTAGTAT
Mouse genotype      ACTATAATTATTTTTTAGTAT
Pig genotype        ACTA-TAATTTTTATTAGTAT
Marsupial genotypeACTA---TATTTTTTTTAGTAT
Dog genotype        ACTA-------TTTATAGTAT
```

FIGURE 1. Sequence alignments of the most variable region of the 18S rRNA gene (positions 689–699) of *Cryptosporidium* isolates from dugongs and other animals.

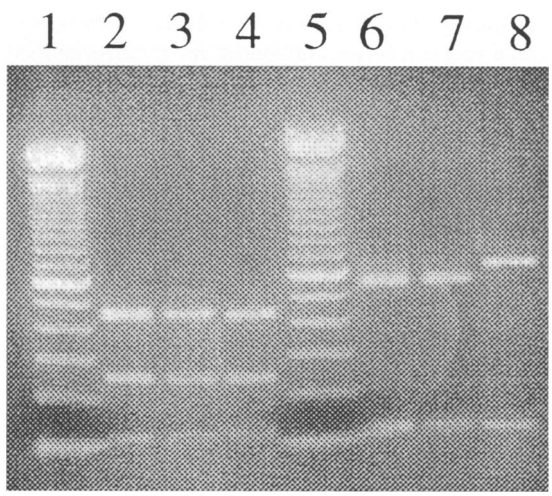

FIGURE 2. A 2.0% agarose gel depicting 18S rDNA PCR/RFLP profiles of human-, bovine-, and dugong-derived *Cryptosporidium* isolates. Lanes 1 and 5: molecular markers (100-bp ladder, Gibco, BRL, Gaithersburg, Maryland); lane 2: dugong sample, *Ssp*I digestion; lane 3: *C. parvum* human genotype, *Ssp*I digestion; lane 4: *C. parvum* bovine genotype, *Ssp*I digestion; lane 6: dugong sample, *Vsp*I digestion; lane 7: *C. parvum* human genotype, *Vsp*I digestion; lane 8: *C. parvum* bovine genotype, *Vsp*I digestion.

The recent report of *Cryptosporidium* in the dugong (*Dugong dugong*) (Hill et al., 1997) is of interest because the dugong is an unusual host and thus may harbor a previously uncharacterized form of *Cryptosporidium*. However, an opportunistic infection resulting from contamination of the water is also possible. The objective of the present study was to characterize the *Cryptosporidium* infection in the dugong using genotyping techniques to provide information on the nature and source of infection.

DNA was extracted from 30-μm sections of paraffin-embedded tissue. Sections were washed in xylene to remove paraffin, and the DNA was extracted using a glass-milk procedure as previously described (Morgan, Pallant, et al., 1998) except that PVPP was omitted from the procedure.

Primers and amplification conditions used to amplify a portion of the gene for the small subunit of ribosomal RNA (rRNA) were as previously described (Morgan, Monis, et al., 1999). TAQ Extender™ (Stratagene, La Jolla, California) was included in all reactions to minimize polymerase chain reaction (PCR) error. PCR products were purified using Qiagen spin columns (Qiagen, Hilden, Germany) and sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions except that the annealing temperature was raised to 60 C. Sequences were analyzed using SeqEd 1.0.3. (Applied Biosystems).

A 390-bp region of the acetyl CoA gene was amplified and sequenced as previously described (Morgan, Sargent, et al., 1998).

Dugong tissue was also analyzed independently by researchers at the Centers for Disease Control (CDC, Atlanta, Georgia). DNA was extracted from sections of paraffin-embedded tissue using the method of Kim et al. (1992). An 837-bp segment of the *Cryptosporidium* 18S rRNA gene was amplified as previously described (Xiao et al., 1999). Sequence data obtained were compared with available *Cryptosporidium* sequences for the same region using the Wisconsin package 9.0 (Genetics Computer Group, Madison, Wisconsin). PCR/restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene was also carried out as previously described (Xiao et al., 1999).

A 374-bp region of the 12S mitochondrial gene was amplified from the dugong tissue using the primers 5′-CATAACGCCTTGCTTAGCC-3′ and 5′-TTGCTAGTAGTTCTCTGGCG-3′. A DNA sample from humans was used as a control. Sequences from the dugong and human were aligned with each other and with those from GenBank using the Wisconsin package.

Sequence and PCR/RFLP analysis of the 18S rRNA gene identified the *Cryptosporidium* in the dugong tissue as having the "human" genotype, i.e., as the *Cryptosporidium* variant usually found in humans. Sequence alignments revealed that the dugong sequence was identical to the recently identified monkey genotype (Xiao et al., 1999), which is considered a variant of the human genotype. Sequence alignments of the most variable region of the 18S rRNA gene (position 689–699) from the dugong isolate and other *Cryptosporidium* isolates is depicted in Figure 1. PCR/RFLP analysis of the 18S rRNA gene from human-, bovine-, and dugong-derived *Cryptosporidium* isolates is depicted in Figure 2.

Sequence analysis of a 390-bp region of the acetyl CoA synthethase gene clearly identified the human genotype in the dugong-derived *Cryptosporidium* sample (GenBank AF254385).

Sequence analysis of a 374-bp portion of the 12S mitochondrial DNA revealed a 1-bp difference from previously published dugong sequence information (GenBank U60185) (Lavergne et al., 1996). Sequence analysis of the human 12S mitochondrial gene revealed 3 or 4 bp differences from existing GenBank sequences. In contrast, the dugong sequence had only 82.8% identity to the human sequence, with 59 substitutions, a 1-bp insertion, and one 5-bp deletion.

To date, 7 distinct genotypes have been identified within what is currently recognized as *C. parvum*: a human genotype found only in humans and rhesus monkeys; a cattle genotype found in ruminants (cattle, sheep, goats, and deer), which appears to be zoonotic because it also has been found in humans; and pig, marsupial, mouse, ferret, and more recently dog genotypes (Morgan, Monis, et al., 1999; Morgan, Xiao, et al., 1999; Xiao et al., 1999).

Sequence analysis at both the18S rDNA and acetyl CoA synthethase loci identified the *Cryptosporidium* human genotype in

DNA extracted from dugong tissue. To rule out PCR contamination, the original block containing the paraffin-embedded tissue was sent to the CDC for independent analysis. Analysis at this laboratory confirmed the identification of the *Cryptosporidium* human genotype using both sequence and PCR/RFLP analysis of the 18S rRNA gene. Sequence analysis of the 12S mitochondrial DNA at this laboratory confirmed the identity of the tissue as dugong in origin.

The *Cryptosporidium* human genotype has previously only been detected in *Cryptosporidium* isolates derived from humans and nonhuman primates. Experimental infection studies have indicated that the *C. parvum* human genotype is not infectious to laboratory animals. This is the first report of this genotype in a nonprimate host. These results may have important public health implications because other mammals may be capable of being infected with this human genotype, thus extending the range of potential reservoirs for human infection. Another possibility is that the infected dugong was immunocompromised and thus more susceptible to infection with this *Cryptosporidium* genotype. Previous studies on individuals infected with the human immunodeficiency virus have revealed that they are susceptible to infection with a range of *Cryptosporidium* species and genotypes (Pieniazek et al., 1999; Morgan et al., 2000). However, because the immune status of the dugongs was not determined, we can only speculate on the susceptibility of dugongs to human genotypes of *Cryptosporidium.*

The dugong examined as part of this study was obtained in the vicinity of the small coastal town of Toogoom, located in Hervey Bay in Queensland, Australia (Hill et al., 1997). Within a 7-day period in 1997, 3 dugongs were reported dead off the coast of this town, and a fourth dugong considered terminally ill was euthanized and necropsied. Tissue sections from this dugong revealed a heavy *Cryptosporidium* infection in the lower small intestine. A range of other tissues examined showed no significant abnormalities. Transmission electron microscopy revealed various life-cycle stages of *Cryptosporidium,* indicating the organism had proliferated in the intestine (Hill et al., 1997). In the original study, oocysts were not identified; however, subsequent analysis by researchers at Murdoch University revealed the presence of putative oocysts, indicating that the parasite may have completed its life cycle in this host and that infectious oocysts were being passed into the surrounding water. Whether this excretion contributed to maintaining the infection in the dugong population is uncertain. A possible source of infection would be human sewage, especially if it contaminated seagrass beds grazed by the dugong. Contamination of the habitat from this source is important because *Cryptosporidium* oocysts are known to survive for long periods in seawater (Fayer et al., 1998; Tamburrini and Pozio, 1999).

The results of the present study also have important implications for dugong populations. Dugongs (also known as sea cows) are marine mammals that are more closely related to elephants than to other marine mammals such as whales and dolphins, but their closest living aquatic relatives are the manatees. Most of the world's dugongs are found in Australian waters between Shark Bay in Western Australia and across the north to Moreton Bay in Queensland. Since 1986–1987, dugong numbers have declined significantly. The decline has been attributed to human causes, e.g., habitat loss, commercial mesh nets (fishing nets), shark nets set for bather protection, and hunting. However, parasite infections from sewage-contaminated waters may also play a role in the decline in dugong numbers. The sensitive ecological status of these animals globally highlights the need for effective management strategies to protect and conserve the Australian population. To assess the threat posed by *Cryptosporidium,* a pilot survey of dugongs utilizing seagrass beds located in heavily populated coastal regions should be conducted. Special attention should be given to locations where seasonal flooding may compromise the function of sewage-treatment facilities. Such a survey would be most informative if it incorporated current technology for genotype analysis.

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