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Identification of a Novel *Cryptosporidium* Genotype in Pigs

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Over a 3-year period, a total of 646 fecal samples from pigs in 22 indoor and outdoor herds from Western Australia were screened for *Cryptosporidium* spp. by microscopy. Results revealed that 39 of 646 samples (6.03%) were positive for *Cryptosporidium*. *Cryptosporidium* was much more common in outdoor herds (17.2%) than in indoor herds (0.5%) and was more common in animals between the ages of 5 and 8 weeks (69.2%) than in younger animals ($P < 0.0001$). Molecular characterization of the positive samples at the 18S ribosomal DNA locus identified two distinct genotypes of *Cryptosporidium*: the previously identified pig genotype I and a novel pig genotype (pig genotype II), both of which warrant species status.

Preweaning diarrhea, caused by a complex of protozoan organisms including *Isospora suis* and *Cryptosporidium* spp., remains a major problem in pigs worldwide (2, 3, 6). Yet, due to the inadequacies of conventional diagnostics, little is known about the prevalence and significance of *Cryptosporidium* in pigs. As a consequence, it has not been possible to adequately determine the contribution of *Cryptosporidium* to porcine preweaning diarrhea. Evidence suggests not only that *Cryptosporidium* is prevalent in domestic pigs but also that *Cryptosporidium* infection can occur concurrently with *Isospora* infection, as well as in older animals (7, 9–11, 14, 16, 17).

Recent genetic characterization studies have revealed that pigs are infected with a genetically distinct and apparently host-adapted form of *Cryptosporidium* (*Cryptosporidium* "pig" genotype) (5, 8, 9, 13). Pigs can also be infected with the zoonotic *Cryptosporidium parvum* "cattle" genotype, indicating that they can potentially play a role as reservoirs of infection for humans and other animals (9). In this study, in order to gain a better understanding of the prevalence and significance of *Cryptosporidium* in pigs, fecal samples were collected over a 3-year period and screened for the presence of *Cryptosporidium* by morphological and molecular analyses.

MATERIALS AND METHODS

Sources of parasite isolates. Fecal samples were collected between August 1999 and July 2001 from indoor and outdoor piggeries in Western Australia. A total of 646 fecal samples from pigs below the age of 9 weeks in 22 (17 indoor and 5 outdoor) herds were screened for the presence of *Eimeria* spp., *I. suis*, *Giardia* spp., and *Cryptosporidium* spp. by direct smear, saturated $ZnSO_4$, and malachite green staining (for the identification of *Cryptosporidium*) (4).

DNA extraction, 18S rDNA gene amplification, and sequencing. DNA was extracted from fecal samples by using the QiAmp stool detection kit (Qiagen, Hilden, Germany). A two-step nested PCR protocol for the 18S ribosomal DNA (rDNA) gene was performed as previously described (19).

PCR products were purified by using Qiagen spin columns and sequenced by using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions, except that the annealing temperature was raised to 58°C. Sequences were analyzed using SeqEd (version 1.0.3; Applied Biosystems).

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Phylogenetic analyses of 18S rDNA. Nucleotide sequences obtained from this study were aligned against each other and those obtained previously by using Clustal X (15). *Plasmodium falciparum* was used as an outgroup (GenBank accession no. M19172). Distance-based analyses were conducted using Tamura-Nei distance estimates, and trees were constructed using the neighbor-joining algorithm (D. L. Swofford, PAUP, 1999). Phylograms were drawn by using the TreeView program (12).

Statistical analysis. Prevalences in different groups of pigs (indoor versus outdoor; different age groups) were compared with a chi-square test for independence.

Nucleotide sequence accession number. A pig genotype II sequence has been submitted to GenBank under accession no. AY271721.

RESULTS

Microscopic analysis. *Cryptosporidium* was detected in 39 of 646 pigs from both indoor and outdoor herds in Western Australia, a prevalence of 6.03% (Table 1). *Cryptosporidium* was significantly more common in outdoor herds (37 of 216 [17.2%]) than in indoor herds (2 of 430 [0.5%]), where only two low-level positive samples were found ($P < 0.0001$). *Cryptosporidium* was found in 1- to 8-week-old pigs but was significantly more common (69.2%) in older animals (5 to 8 weeks old) than in pigs below the age of 5 weeks ($P < 0.0001$). This contrasts with bovine hosts, where cryptosporidiosis is most common in calves 2 weeks old or younger (11).

In the majority of samples the number of oocysts detected was low (<1,000/g of feces), with the exception of four samples, all from the same herd (PGl 1, PGl 7, PG1 8, and PGl 31), where oocyst numbers of $>10^6$/g of feces were detected (Table 1). A number of other parasites were detected, including *I. suis* (15%), a *Giardia* sp. (0.8%), *Ascaris suum* (0.6%), and *Eimeria* spp. (18%). In some cases multiple parasite infections were detected; for example, isolates PGl 22 to 25 were positive for *Cryptosporidium*, a *Giardia* sp., and *Eimeria* spp.

Sequencing and phylogenetic analysis of the 18S ribosomal DNA (rDNA) gene. Partial sequences were obtained for 28 of 39 pig-derived *Cryptosporidium* isolates (Table 1). The remaining 11 isolates either could not be amplified or produced mixed chromatograms, which could not be read. Sequencing and phylogenetic analysis of the 28 pig-derived isolates and a range of *Cryptosporidium* 18S sequences obtained from GenBank identified two distinct genotypes in pigs: the previously identified *Cryptosporidium* "pig" genotype (9) and a novel genotype,

TABLE 1. *Cryptosporidium* isolates used in this study

Isolate	Age of host (wks)	I/O herd[a]	Toltrazuril treatment[b]	No. of oocysts/g of feces	Date collected (day/mo/yr)	Genotype at 18S rDNA locus[c]
PW 1	4–6	O	Y	<1,000	16/09/99	ND
PW 2	6–8	O	Y	<1,000	16/09/99	Pig genotype II
PW 3	6	O	Y	<1,000	16/09/99	Pig genotype II
PW 4	5	O	Y	<1,000	16/09/99	Pig genotype II
PM3-2	4	I	N	<1,000	21/09/99	Pig genotype II
PM3-3	4	I	N	<1,000	21/09/99	ND
PGR- 1	4	O	N	<1,000	23/12/99	ND
PGR- 2	4	O	N	<1,000	23/12/99	ND
PGI 1	1	O	N	1.1×10^6	25/07/99	Pig genotype II
PGI 2	2	O	N	<1,000	25/07/99	ND
PGI 5	2	O	Y	7×10^5	25/07/00	Pig genotype I
PGI 7	2	O	Y	9.6×10^6	25/07/00	ND
PGI 8	2	O	Y	7×10^6	25/07/00	Pig genotype I
PGI 9	4	O	N	<1,000	25/07/00	Pig genotype I
PGI 11	5	O	N	<1,000	25/07/00	ND
PGI 13	5	O	U	<1,000	25/07/00	Pig genotype I
PGI 14	5	O	U	6.8×10^5	25/07/00	ND
PGI 16	5	O	U	<1,000	25/07/00	Pig genotype I
PGI 18	6	O	U	≈1,000	25/07/00	Pig genotype II
PGI 19	6	O	U	1.2×10^5	25/07/00	Pig genotype II
PGI 20	6	O	U	2×10^5	25/07/00	Pig genotype II
PGI 22	6	O	U	<1,000	25/07/00	Pig genotype II
PGI 23	6	O	U	2×10^4	25/07/00	Pig genotype I
PGI 24	6	O	U	6×10^5	25/07/00	Pig genotype I
PGI 25	6	O	U	<1,000	25/07/00	Pig genotype II
PGI 26	6	O	U	<1,000	25/07/00	Pig genotype II
PGI 28	8	O	U	<1,000	25/07/00	Pig genotype II
PGI 29	8	O	U	<1,000	25/07/00	ND
PGI 31	8	O	U	1.5×10^6	25/07/00	Pig genotype II
POR10	6	O	N	<1,000	25/07/00	Pig genotype I
POR12	5	O	N	<1,000	25/07/00	ND
POR21	8	O	N	<1,000	25/07/00	Pig genotype II
POR32	2	O	N	<1,000	25/07/00	Pig genotype I
POR33	2	O	N	<1,000	25/07/00	Pig genotype I
POR 3	6	O	N	<1,000	31/07/01	Pig genotype I
PMa 4	7	O	N	<1,000	31/07/01	ND
PMa12	7	O	N	<1,000	31/07/01	Pig genotype I
PMa23	7	O	N	<1,000	31/07/01	Pig genotype I
PMa28	7	O	N	<1,000	31/07/01	Pig genotype I

[a] I/O, indoor or outdoor.
[b] Y, yes; N, no; U, unknown.
[c] ND, not determined.

which we have called *Cryptosporidium* "pig" genotype II (Fig. 1 and 2). In the samples that could be genotyped, pig genotype I and pig genotype II appeared to be present in equal numbers (i.e., each was present in 14 of 28 samples [50%]). There was no difference in age between pigs infected with genotypes I and II. Some pigs infected with pig genotype II did appear to shed high numbers of oocysts, but more-intensive sampling is required to confirm this.

DISCUSSION

Microscopic analysis of fecal samples from indoor and outdoor piggeries in Western Australia identified *Cryptosporidium* in 39 of 646 pigs tested (6.03%); most of these samples were not associated with diarrhea. A previous study of indoor and outdoor piggeries in Western Australia detected the presence of both the pig genotype and the *C. parvum* cattle genotype (9). That study concluded that "two genetically and biologically differing strains of *Cryptosporidium* appeared to be associated with acute diarrhea, chronic ill thrift and death in pigs" (9). However, it was not possible to determine whether *Cryptosporidium* was the primary or the secondary pathogen, because no screening for viruses and enteric bacteria was performed. Similarly, in the present study, it was not possible to determine if *Cryptosporidium* was the primary pathogen, but in most cases, infection with *Cryptosporidium* was not associated with diarrhea, suggesting that both pig genotype I and pig genotype II are host adapted. Further studies are required to confirm this hypothesis.

Few studies have been conducted in recent years to determine the prevalence of *Cryptosporidium* in pigs. A study in Japan reported a much higher prevalence of *Cryptosporidium* in pigs, with 77 (33.2%) 1- to 3-month-old weaned piglets positive for *Cryptosporidium* from four out of eight stock-raising farms located in Kanagawa Prefecture (7). In another study, in Germany, *Cryptosporidium* was detected in 1.4% of piglets, all of which exhibited diarrhea (16). More-extensive surveys need to be performed, but it is clear that cryptospo-

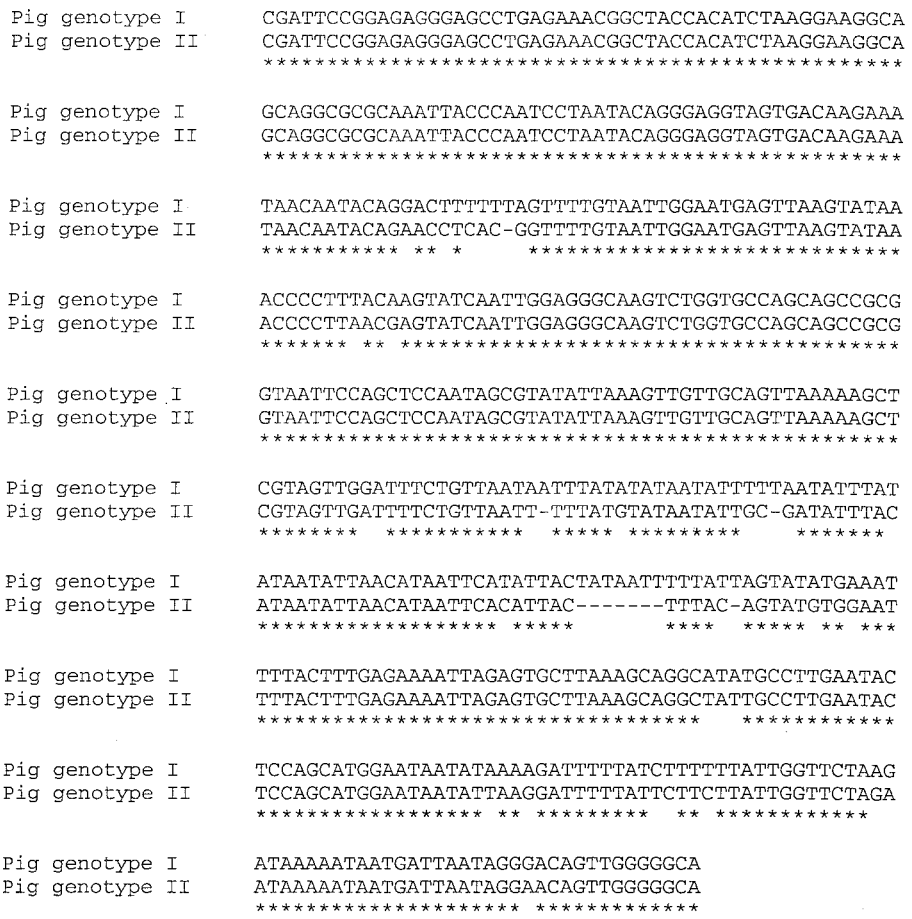

FIG. 1. Clustal X sequence alignment of partial 18S rDNA sequences of pig genotype I and pig genotype II.

ridial infection, perhaps in combination with other enteric pathogens, may be an emerging problem for the pig industry.

In the present study, the prophylactic use of toltrazuril at the age of 3 to 5 days was widespread (384 of 646 samples), particularly in the indoor intensive farms. This is interesting in light of the fact that *Cryptosporidium* was much more common in outdoor herds (37 of 216 [17.2%]) than in indoor herds (2 of 430 [0.5%]). A study by Armson et al. (1) has shown that toltrazuril exhibited limited efficacy against the *C. parvum* cattle genotype in vitro, but it has not been tested in vivo. It is possible that the extensive prophylactic use of toltrazuril in indoor herds inhibited the establishment of *Cryptosporidium* infections in these pigs. However, high numbers of oocysts were detected in two isolates from an outdoor herd which had been prophylactically treated with toltrazuril (Table 1). Further studies are required to confirm the effect, if any, of toltrazuril on *Cryptosporidium*.

A more likely explanation is that the opportunities for transmission of *Cryptosporidium* are much greater in outdoor herds through the contamination of the environment to which the pigs have access. In the indoor herds the pigs were maintained in pens with slatted flooring that allowed fecal matter to fall through. The slats were rinsed off daily, thus limiting any potential exposure to contaminated feces. This theory is supported by a previous study on the prevalence of *Cryptosporidium* in two Ohio pig farms with different management systems (18). That study reported that a farm with porous concrete floors had a significantly higher *Cryptosporidium* infection rate in pigs than a farm with slotted and wire floors (18).

Sequencing and phylogenetic analysis of 18S rDNA identified two distinct genotypes of *Cryptosporidium*: the previously identified pig genotype (9) and a novel pig genotype (pig genotype II). The previous study by Morgan et al. (9) reported the presence in pigs of the *C. parvum* cattle genotype, which is associated with zoonotic transmission. However, in the present study, the cattle genotype was not detected, which argues against the previous speculation that pigs may be important reservoirs of zoonotic *Cryptosporidium* in Australia (9).

Phylogenetic analysis of the 18S locus revealed that *Cryptosporidium* pig genotype I and pig genotype II are genetically distinct. Pig genotype I shared only 97.3% similarity with the *C. parvum* cattle genotype and *C. hominis*. This is less than the similarities among *C. meleagridis*, the *C. parvum* cattle genotype, and *C. hominis* (99 to 98.6%) and between the *C. parvum* cattle genotype and *C. hominis* (99.2%). *Cryptosporidium* pig genotype II is genetically very distinct and did not group within the *C. parvum* genotypes or closely related species but formed a separate clade on its own. *Cryptosporidium* pig genotype II shared only 93% similarity with pig genotype I and only 92.2 to

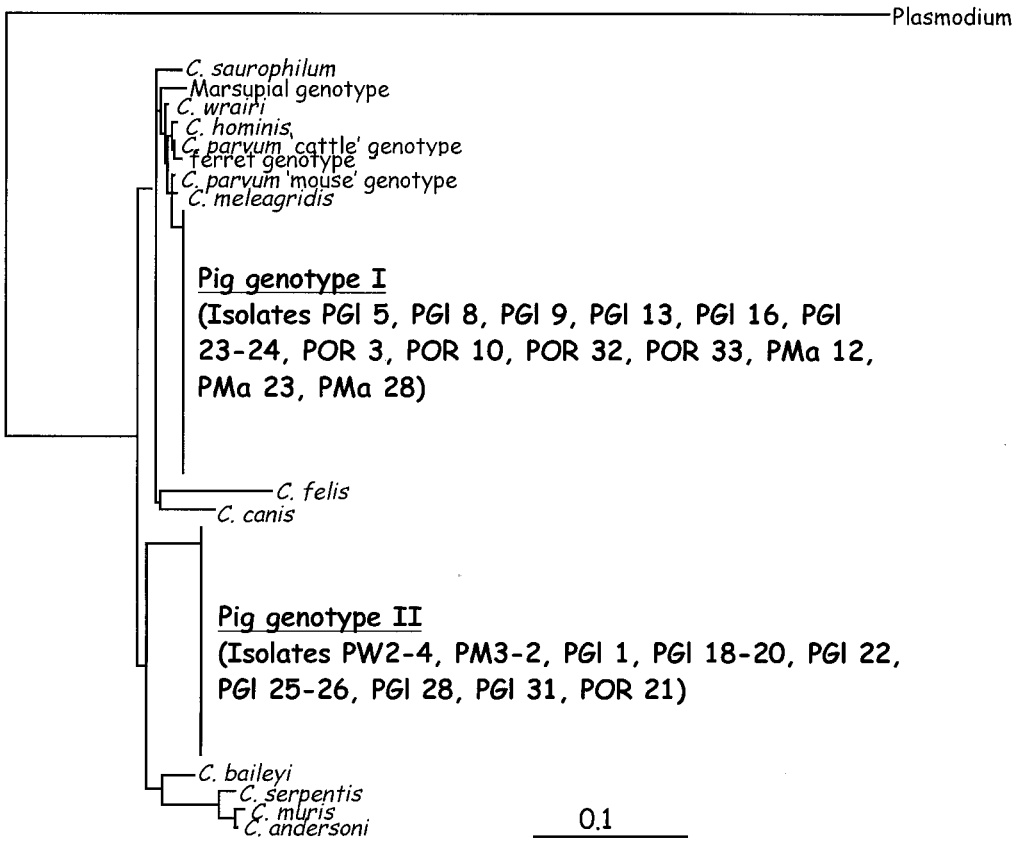

FIG. 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura-Nei distances calculated from pairwise comparisons of the 18S rDNA sequences.

92.8% similarity with the *C. parvum* cattle genotype, the *C. parvum* "mouse" genotype, *C. hominis*, and *C. meleagridis*. This is similar to the level of genetic similarity between the *C. parvum* group and *C. baileyi* (93.7%) and between the *C. parvum* group and *C. muris*, *C. serpentis*, and *C. andersoni* (87.7 to 89%), which are the *Cryptosporidium* species most distantly related to the *C. parvum* group

Both pig genotypes appear to be widespread, since both were first identified in pig-derived isolates from Switzerland (8; U. M. Ryan, unpublished data) and have since been identified in other locations in Europe (5; L. Xiao et al., unpublished data). The genetically distinct nature of both these genotypes, combined with their apparently host-adapted nature, strongly suggests that both pig genotype I and the novel pig genotype II warrant species status.

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