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Subtype analysis of zoonotic pathogen *Cryptosporidium* skunk genotype

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Abstract

Cryptosporidium skunk genotype is a zoonotic pathogen commonly identified in surface water. Thus far, no subtyping tool exists for characterizing its transmission in humans and animals and transport in environment. In this study, a subtyping tool based on the 60 kDa glycoprotein (gp60) gene previously developed for *Cryptosporidium* chipmunk genotype I was used in the characterization of *Cryptosporidium* skunk genotype in animal and storm runoff samples from a watershed in New York. Altogether, 17 positive samples from this watershed and 5 human and animal specimens from other areas were analyzed. We identified 14 subtypes of *Cryptosporidium* skunk genotype, 11 of which were seen in the watershed. In phylogenetic analysis, these subtypes belonged to 4 subtype families (XVIa, XVIb, XVIc, and XVId). No host-adapted subtypes were identified and the two subtypes in humans were genetically similar to some in raccoons, otters, and storm runoff samples from the watershed. The characteristics of gp60 protein sequences of the *Cryptosporidium* skunk genotype are similar to those of other *Cryptosporidium* species, but only its XVIb subtype family has a putative furin cleavage site. This subtyping tool might be useful in characterizing *Cryptosporidium* skunk genotype in clinical and environmental samples.

Keywords

Cryptosporidium; gp60; Subtyping; Zoonosis; One health; Water

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1. Introduction

Cryptosporidiosis is a major waterborne disease in both industrialized and developing countries (Checkley et al., 2015). *Cryptosporidium hominis* and *C. parvum* are the leading causes of cryptosporidiosis in humans globally (Ryan et al., 2014; Xiao, 2010). However, some *Cryptosporidium* species or genotypes from wildlife, including *C. ubiquitum*, *C. cuniculus*, *Cryptosporidium* chipmunk genotype I, and skunk genotype, have been detected in humans in some areas (Davies et al., 2009; Elwin et al., 2012; Feltus et al., 2006; Lebbad et al., 2013; Robinson et al., 2008). Subtyping these unusual species or genotypes would facilitate the tracking of their environmental contamination.

Cryptosporidium skunk genotype was initially isolated from striped skunks in California, USA and was named this way because of the presumed host specificity of most *Cryptosporidium* spp. (Xiao et al., 2002). It, however, has been subsequently identified in other wild mammals including raccoons, eastern gray squirrels, American red squirrels, fox squirrels, river otters, Virginia opossums, and southern elephant seals (Feng et al., 2007; Rengifo-Herrera et al., 2011; Stenger et al., 2015b; Zhou et al., 2004). Because of its broad host range, *Cryptosporidium* skunk genotype is common in surface water in the United States and Canada (Jellison et al., 2009; Jiang et al., 2005; Ruecker et al., 2012; Yang et al., 2008), and has been identified in five human cases in the United States and United Kingdom (Davies et al., 2009; Robinson et al., 2008). Currently, there are no subtyping tools for this zoonotic parasite, which makes it difficult to investigate its transmission in humans (Robinson et al., 2008; Xiao, 2010).

Subtyping tools are widely used in the characterization of transmission and environmental transport of *C. parvum* and *C. hominis* (Ryan et al., 2014; Xiao, 2010). One common target used in subtyping of these species is the 60-kDa glycoprotein (gp60) gene, as it is highly polymorphic and divides the two *Cryptosporidium* species into several major subtype families (Strong et al., 2000). In recent years, subtyping tools based on gp60 sequences have been developed for other *Cryptosporidium* species and genotypes such as *C. meleagridis*, *C. ubiquitum*, *C. fayeri*, *C. viatorum*, and *Cryptosporidium* chipmunk genotype I (Guo et al., 2015; Li et al., 2014; Power et al., 2009; Stensvold et al., 2014; Stensvold et al., 2015). With the availability of whole genome sequences, some of the new tools, such as the one for *Cryptosporidium* chipmunk genotype I, have used conserved nucleotide sequences in PCR primer design (Guo et al., 2015). The latter was shown to be able to detect *C. ubiquitum* and possibly *Cryptosporidium* skunk genotype in water samples in addition to *Cryptosporidium* chipmunk genotype I (Guo et al., 2015). In this study, we have used this tool in subtyping *Cryptosporidium* skunk genotype present in wildlife, storm runoff and three humans.

2. Materials and methods

2.1. Specimens

Genomic DNA preparations from 22 samples of *Cryptosporidium* skunk genotype were used in the study, including six from raccoons, one from eastern gray squirrel, and one from river otter in a watershed in New York, USA, nine from storm runoff collected from creeks in the same watershed, two from striped skunks in California, one from a human in Nebraska, and

two from humans in the United Kingdom (Table 1). All three human specimens were from patients who sought medical care because of the occurrence of diarrhea. These specimens were identified as positive for *Cryptosporidium* skunk genotype by PCR and sequence analysis of the small subunit (SSU) rRNA gene (Xiao et al., 2002).

2.2. PCR analysis of gp60 gene

The gp60 gene of *Cryptosporidium* skunk genotype was amplified from each DNA preparation using nested-PCR. The previously described Chip-F1 (5′ TTTACCCACACATCTGTAACGTCG 3′) and Chip-R1 (5′ CCTGTGAGAATATTCTGGAAATTA 3′) and Chip-F2 (5′ ATAGGTAATAATTACTCAGTATTTAAT 3′) and Chip-R2 (5′ TCATCTTAAAACGCTTAAACTCTTAA 3′) primers for *Cryptosporidium* chipmunk genotype I were used in primary and secondary PCR, respectively (Guo et al., 2015). Duplicate PCR reactions were used in the analysis of DNA from human and animal specimens whereas quintuplicate PCR reactions were used in the analysis of DNA from storm runoff samples. The PCR condition in this study was identical to the one described previously (Guo et al., 2015), except that the annealing temperature in both primary and secondary PCR was changed from 55 °C to 52 °C. The secondary PCR products were analyzed by 1.5% agarose gel electrophoresis.

2.3. Sequence analysis

Positive PCR products were sequenced using the Chip-F2 and Chip-R2 primers in both directions on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Due to the likely presence of multiple *Cryptosporidium* species or subtypes (Jiang et al., 2005), all PCR products from storm runoff samples were sequenced. The nucleotide sequences obtained were assembled using ChromasPro 1.7.7 (http://technelysium.com.au/wp/chromaspro/), edited using BioEdit 7.04 (www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned with each other and reference sequences from the GenBank database using ClustalX2.1 (www.clustal.org/). The subtype families and subtypes were named using the established gp60 subtype nomenclature (Ryan et al., 2014).

To assess the uniqueness of the gp60 protein of *Cryptosporidium* skunk genotype, the nucleotide sequences obtained were translated into amino acid sequences using the program EditSeq in DNASTAR Lasergene 12.3.1 (http://www.dnastar.com/t-dnastar-lasergene.aspx). Signal peptide was predicted using SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/). The C-terminal glycosylphosphatidylinositol (GPI) anchor and transmembrane domain were predicted using the program Protean in DNASTAR Lasergene 12.3.1, PredGPI (http://gpcr2.biocomp.unibo.it/gpipe/pred.htm) and PSORT II (http://psort.hgc.jp/form2.html), respectively. The predictions of potential N-glycosylation and O-glycosylation sites were performed using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively. These sequences were also analyzed for the presence of furin cleavage sites using the ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/).

To assess the genetic relationship among gp60 sequences from the *Cryptosporidium* skunk genotype and other *Cryptosporidium* species and genotypes, a maximum likelihood tree was constructed using Tamura-Nei evolutionary distances calculated in MEGA 7.0.7 (http://www.megasoftware.net/). DnaSP 5.10 (www.ub.es/dnasp/) was used to calculate recombination rates among various subtype families of *Cryptosporidium* skunk genotype and chipmunk genotype I.

2.4. 3.4. Nucleotide sequence accession numbers

Nucleotide sequences of the gp60 gene of *Cryptosporidium* spp. generated in this study were deposited in GenBank under the accession numbers KX698285 to KX698307.

3. Results

3.1. PCR analysis of gp60 gene in Cryptosporidium skunk genotype

Using the previously described primers for *Cryptosporidium* chipmunk genotype I (Guo et al., 2015), the gp60 gene was efficiently amplified for all 22 DNA samples. For all human and animal specimens, both replicate PCR were positive, compared with one to five replicates positive for storm runoff samples. DNA sequencing results showed that the gp60 sequences from 19 samples were similar to KP099095 from *Cryptosporidium* skunk genotype, with sequence lengths of 1035–1109 bp. However, sequences generated from the remaining three storm runoff samples were identical to *C. ubiquitum* XIIb or XIId subtype family (XIIb in one PCR replicate each from samples 8651, 8514 and 6858; and XIId in four PCR replicates from sample 8651 and one replicate from sample 8514).

3.2. Subtypes of Cryptosporidium skunk genotype in humans, wild animals, and storm runoff

Four major types of nucleotide sequences of the gp60 gene were obtained from the *Cryptosporidium* skunk genotype in this study. These types of sequences differed significantly among each other in the non-repeat regions of the gene, while within each major type sequences differed from each other mostly in the copy number of the TCA or TCG repeats. According to the established gp60 subtype nomenclature, these four major types of *Cryptosporidium* skunk genotype sequences were named as XVIa, XVIb, XVIc, and XVId subtype families. There were three subtypes within the subtype family XVIa, five in XVIb, four in XVIc, and two in XVId, resulting in 14 subtypes among these 19 samples of the *Cryptosporidium* skunk genotype. One human case from Nebraska, USA was identified as subtype XVIcA22, and two human cases from the UK were identified as subtype XVIbA16G2b (Table 1). These two subtypes were not seen in wildlife or storm runoff samples in New York.

There were six subtypes of the *Cryptosporidium* skunk genotype in storm runoff samples from the New York watershed. Similarly, there were six subtypes of the *Cryptosporidium* skunk genotype in animal specimens from the same watershed. One of the subtypes, XVIbA16G2a, occurred in both storm runoff and animals from the same watershed, and one water sample (No. 8649) had two XVIb subtypes (Table 1). Therefore, there were a total of 11 subtypes of the *Cryptosporidium* skunk genotype in the watershed. Subtype

XVIbA16G2a had one synonymous substitution (T → C at nucleotide 1029 nt) in the non-repeat region compared with subtype XVIbA16G2b.

A maximum likelihood tree was constructed with 19 gp60 gene sequences of the *Cryptosporidium* skunk genotype generated in this study, one *Cryptosporidium* skunk genotype sequence previously published (KP099095), and GenBank sequences from several other *Cryptosporidium* species or genotypes (Fig. 1). All 20 sequences of the *Cryptosporidium* skunk genotype clustered into one large clade that appeared to be a sister to *Cryptosporidium* chipmunk genotype I. Other *Cryptosporidium* species or genotypes, in contrast, were more distant (Fig. 1). In agreement with direct sequence comparison, nucleotide sequences of the *Cryptosporidium* skunk genotype formed four subclades in phylogenetic analysis. The mean genetic distances in nucleotide sequences were 0.232–0.392 substitution/site between subtype families and 0–0.0021 within each subtype family.

DnaSP analysis of the gp60 nucleotide sequences revealed the occurrence of 37 recombination events across the gene among four subtype families of *Cryptosporidium* skunk genotype (XVIa-XVId), but only two recombination events if only XVIa, XVIb, and XVIc sequences were included in the analysis. Pairwise recombination event comparisons revealed the presence of one probable genetic recombination between subtype families XVIb and XVId (Table 2).

3.3. Characteristics of gp60 gene of Cryptosporidium skunk genotype

The complete open reading frame (ORF) of the gp60 gene was obtained for 15 of the 19 *Cryptosporidium* skunk genotype sequences generated in this study. One full sequence of the gp60 from specimen 42,693 is used here to elucidate the characteristics of gp60 gene in *Cryptosporidium* skunk genotype. The ORF of the gp60 gene consists of 1089 nucleotides, encoding a peptide of 362 amino acids. Except for the 5′ and 3′ regions, the gp60 gene of *Cryptosporidium* skunk genotype is significantly different from those of *C. hominis*, *C. parvum*, *C. ubiquitum*, chipmunk genotype I and other species or genotypes, with only 40.2% to 72.0% of nucleotide sequence identity. The gp60 protein of *Cryptosporidium* skunk genotype has a signal peptide in the first 18 amino acids with a cleavage site between amino acids Ser_{18} and Ala_{19}. Following the signal peptide, the deduced amino acid sequence contains one polyserine track including 18 Ser residues, which were predicted to be O-glycosylation sites. Several other Ser and Thr residues throughout the sequence were predicted to be O-glycosylation sites. Two potential *N*-glycosylation sites were identified in the gp60 protein sequence. A hydrophobic region is present at the C-terminus of the gp60 protein, and is linked to a GPI anchor. However, no transmembrane domain is present in the gp60 protein sequence. In addition, the predicted furin cleavage site sequence RVAR in the XVIb subtype family is different from the conserved sequence RSRR in *C. hominis* and *C. parvum* (Guo et al., 2015; Stensvold et al., 2015). No furin cleavage site was found in the other three subtype families (XVIa, XVIc and XVId) of *Cryptosporidium* skunk genotype (Fig. 2).

4. Discussion

In this study, we have shown that gp60 PCR primers we previously designed for *Cryptosporidium* chipmunk genotype I can efficiently amplify the gp60 gene of *Cryptosporidium* skunk genotype in water samples and stool specimens. We hypothesized that because *Cryptosporidium* skunk genotype is genetically related to *Cryptosporidium* chipmunk genotype I at the SSU rRNA and 70 kDa heat shock protein loci (Feng et al., 2007; Lv et al., 2009; Stenger et al., 2015a), gp60 PCR primers designed for *Cryptosporidium* chipmunk genotype I could amplify the gp60 gene of *Cryptosporidium* skunk genotype. In addition, the nested-PCR primers for subtyping *Cryptosporidium* chipmunk genotype I were designed based on conserved nucleotide sequences flanking the gp60 gene of *C. parvum*, *C. hominis*, *C. ubiquitum*, and *Cryptosporidium* chipmunk genotype I (Guo et al., 2015). It is well known that because of the extensive sequence differences in the gp60 gene among various *Cryptosporidium* species and genotypes, PCR primers based on *C. parvum* and *C. hominis* sequences alone generally fail to amplify the gp60 gene of other *Cryptosporidium* species and genotypes that are genetically distant from these two species (Feng et al., 2011; Xiao, 2010). Phylogenetic analysis of gp60 sequences obtained in this study has shown a close genetic relatedness between *Cryptosporidium* skunk genotype and *Cryptosporidium* chipmunk genotype I, reinforcing the usefulness of the *Cryptosporidium* chipmunk genotype I primers in PCR analysis of the gp60 gene of *Cryptosporidium* skunk genotype.

The detection of *C. ubiquitum* gp60 sequences in some storm runoff samples in addition to those from *Cryptosporidium* skunk genotype supports the broad specificity of the gp60 PCR primers used in this study. As storm runoff samples frequently contain mixed *Cryptosporidium* species/genotypes (Xiao et al., 2006), three of the nine samples analyzed produced *C. ubiquitum* gp60 sequences, in agreement with the result of SSU rRNA-based PCR-sequencing analysis of the samples. These three runoff samples had 1 and 2, 1 and 4, and 2 and 2 replicates positive for *Cryptosporidium* skunk genotype and *C. ubiquitum* in quintuplicate PCR, respectively. Previously, using these *Cryptosporidium* chipmunk genotype I primers, we were able to amplify the gp60 gene of *C. ubiquitum* in some storm runoff samples (Guo et al., 2015). In one of the samples analyzed (KP099095), a nucleotide sequence representing a new gp60 subtype family was obtained. As the sample had concurrent presence of *Cryptosporidium* skunk genotype, it was suggested that the gp60 sequence could be from this parasite (Guo et al., 2015). As a result, a new subtype family name XVIa was designated. Here, we detected similar XVIa sequences in one eastern squirrel specimen and one storm runoff sample that are known to have *Cryptosporidium* skunk genotype based on SSU rRNA-based PCR-RFLP and sequence analyses (Table 1).

A significant genetic heterogeneity is apparently present in *Cryptosporidium* skunk genotype. In this study, in addition to XVIa, three other subtype families of gp60 sequences were detected in humans, animals, and storm runoff samples. According to the established gp60 subtype nomenclature, they were named as XVIb, XVIc, and XVId. Genetic recombination, as suggested in other *Cryptosporidium* species (Li et al., 2014), could play a potential role in the generation of high genetic heterogeneity in *Cryptosporidium* skunk genotype, as potential genetic events were detected among some of the subtype families in

this study. This is especially the case in subtype family XVId, which has very high nucleotide sequence similarity to XVIc (Fig. 1 and Table 2). Recombinant event analysis indicates that it could be a recombinant between XVIc and XVIb subtype families.

Unlike in some *Cryptosporidium* species such as *C. parvum*, *C. tyzzeri*, and *C. ubiquitum* in which host adaptation exists among subtype families (Kvac et al., 2013; Li et al., 2014; Xiao, 2010), there might be no apparent host-adaptation among subtype families of *Cryptosporidium* skunk genotype based on the characterization of specimens from several animal species. In fact, all four subtype families described in this study were found in animals and storm runoff in the New York watershed. Altogether, 14 subtypes in these four subtype families were seen in humans, raccoons, skunks, river otter, east gray squirrel and storm runoff. However, most of the animal specimens were from carnivores, which has prevented full assessment of host adaptation. Among the 14 subtypes described in this study, 11 were detected in animal and storm runoff samples from the New York watershed examined. Two subtypes (XVIbA16G2b and XVIcA22) were detected in humans. The XVIb subtype family appears to be the most common one, being detected in humans, various animals, and storm runoff. In particular, its XVIbA16G2b subtype detected in two human cases in the United Kingdom is very similar to the XVIbA16G2a subtype, which was found in raccoons and storm runoff samples in the United States and has only one synonymous nucleotide substitution (T → C) in the gp60 gene. Therefore, at least XVIb and XVIc subtype families of *Cryptosporidium* skunk genotype could be human pathogens. More extensive characterizations of human and animal specimens are needed to understand host specificity and cross-species transmission of *Cryptosporidium* skunk genotype.

The close genetic relatedness between *Cryptosporidium* skunk genotype and *Cryptosporidium* chipmunk genotype I is interesting. Data obtained thus far indicate that the former is mostly a pathogen of Carnivora (Table 1) whereas the latter is mostly a parasite of Rodentia and Eulipotyphla (Feng et al., 2007; Guo et al., 2015; Kvac et al., 2008; Lv et al., 2009; Song et al., 2015). The occurrence of both in humans as pathogens indicates that both *Cryptosporidium* genotypes have much broad host ranges than the names have indicated. The current practice in naming new *Cryptosporidium* genotypes after their initial hosts has some obvious drawbacks. Although there is clear host adaptation among most *Cryptosporidium* spp., no *Cryptosporidium* species or genotypes are known to have absolute host specificity and some of them, such as *C. parvum* and *C. ubiquitum*, are known to have a broad host range. Designating species names to these *Cryptosporidium* genotypes upon extensive biologic and genetic characterizations would alleviate some of the confusions.

The gp60 gene of *Cryptosporidium* skunk genotype has typical characteristics of the gene seen in other *Cryptosporidium* species, including a signal peptide in the first 18 amino acids, a polyserine track, various O-glycosylation sites and two N-glycosylation sites, and a GPI anchor at the C-terminus. Interestingly, only the XVIb subtype family of this parasite has a furin cleavage site, which is needed for the cleavage of the gp60 precursor protein into gp15 and gp40 by a subtilisin-like protease during the invasion process (Wanyiri et al., 2009). *Cryptosporidium* chipmunk genotype I also does not have the classic furin cleavage sequence RSRR in its gp60 gene. Instead, the sequence was replaced by LSKR, which may or may not be cleaved by the subtilisin protease (Guo et al., 2015). Neither RSRR nor LSKR

sequences are present in the gp60 gene of XVIa, XVIc, and XVId subtype families of *Cryptosporidium* skunk genotype, indicating that the gp60 protein may be processed differently in these parasites. The gp60 gene of *C. ubiquitum* also does not appear to have any furin cleavage site (Li et al., 2014). Further studies are needed to examine the significance of the lack of a furin cleavage site in gp60 protein in *Cryptosporidium* invasion.

In summary, the gp60 primers described previously for *Cryptosporidium* chipmunk genotype I can be used efficiently in subtyping *Cryptosporidium* skunk genotype in animal and storm runoff samples from watershed. A preliminary application of this tool in analysis of stool specimens and water samples indicates the presence of heterogeneous populations of the parasite in a small geographic area. Further studies are needed to improve our knowledge on the environmental ecology and transmission of this zoonotic pathogen.

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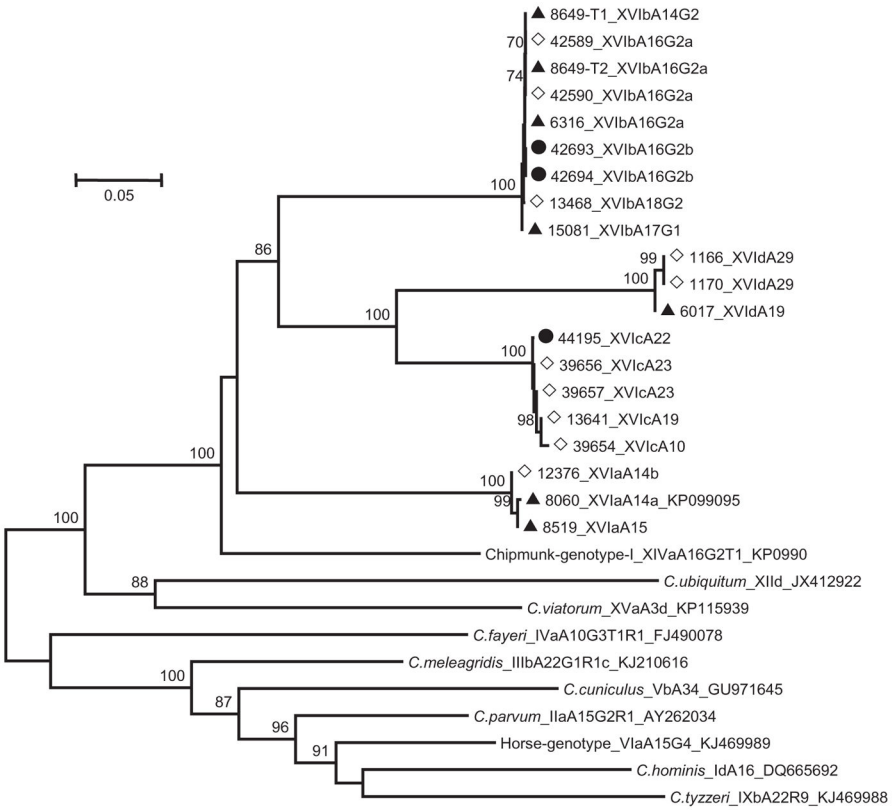

Fig. 1.

Phylogenetic relationship among *Cryptosporidium* skunk genotype and some other published *Cryptosporidium* species and genotypes in GenBank based on the maximum likelihood analysis of nucleotide sequences of the gp60 gene. Numbers on branches are percent bootstrapping values (> 50) using 1000 replicates. ◇: isolate from wildlife including skunks, raccoons, river otter and eastern gray squirrel; ▲: isolate from storm runoff; ●: isolate from humans.

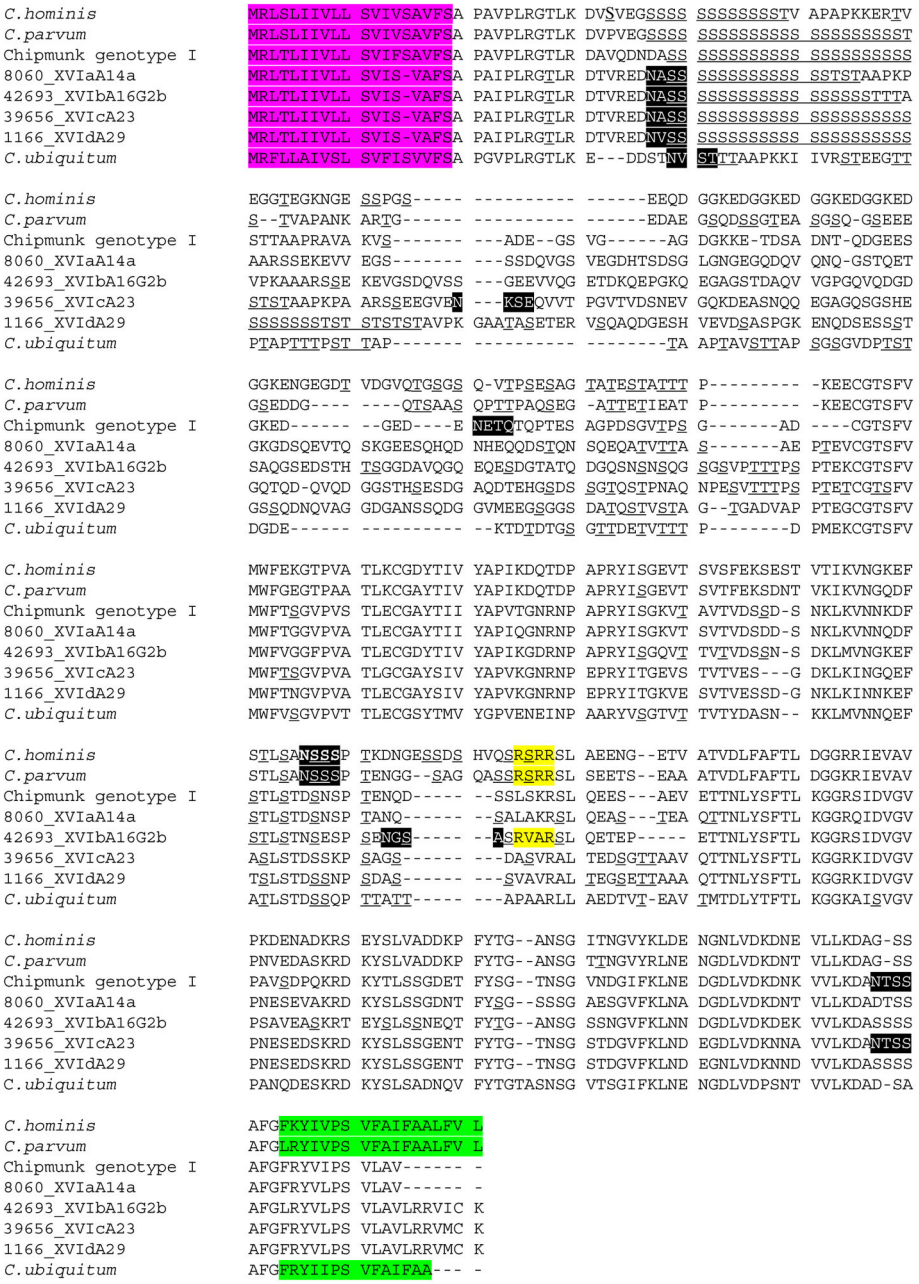

Fig. 2.

Alignment of gp60 protein sequences of *Cryptosporidium* skunk genotype compared with *C. hominis* (ACQ82748), *C. parvum* (AF022929), *Cryptosporidium* chipmunk genotype I (AJW72309), and *C. ubiquitum* (AJW72317). The signal peptides in the amino acid sequences are shaded in pink, the transmembrane domains in green, the furin cleavage sites in yellow, potential *N*-glycosylation sites in black, and O-glycosylation sites are underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Cryptosporidium specimens used in this study and their subtype identifications based on sequence analysis of the gp60 gene.

Source	Sample ID	Year of collection	Location of collection	SSU rRNA genotype	gp60 subtype**	Reference
Human	UKSK1 (42694)	2010	East Midlands, UK	Skunk genotype	XVIbA16G2b	This study
Human	UKSK2 (42693)	2013	South West, UK	Skunk genotype	XVIbA16G2b	This study
Human	44,195	2016	Nebraska, USA	Skunk genotype	XVIcA22	This study
Skunk	1166	1999	California, USA	Skunk genotype	XVIdA29	Xiao et al., 2002
Skunk	1170	1999	California, USA	Skunk genotype	XVIdA29	Xiao et al., 2002
Eastern gray squirrel	12,376	2006	New York, USA	Skunk genotype	XVIaA14b	Feng et al., 2007
River otter	13,641	2006	New York, USA	Skunk genotype	XVIcA19	Feng et al., 2007
Raccoon	13,468	2006	New York, USA	Skunk genotype	XVIbA18G2	Feng et al., 2007
Raccoon	42,589	2015	New York, USA	Skunk genotype	XVIbA16G2a	This study
Raccoon	42,590	2015	New York, USA	Skunk genotype	XVIbA16G2a	This study
Raccoon	39,654	2013	New York, USA	Skunk genotype	XVIcA10	This study
Raccoon	39,656	2013	New York, USA	Skunk genotype	XVIcA23	This study
Raccoon	39,657	2013	New York, USA	Skunk genotype	XVIcA23	This study
Runoff	8060	2003	New York, USA	Skunk genotype	XVIaA14a	Jiang et al., 2005
Runoff	6017	2002	New York, USA	Skunk genotype	XVIdA19	Jiang et al., 2005
Runoff	8649*	2003	New York, USA	Skunk genotype	XVIbA14G2, XVIbA16G2a	Jiang et al., 2005
Runoff	15,081	2007	New York, USA	Skunk genotype	XVIbA17G1	This study
Runoff	8519	2003	New York, USA	Skunk genotype	XVIaA15	Jiang et al., 2005
Runoff	6316	2002	New York, USA	Skunk genotype	XVIbA16G2a	Jiang et al., 2005
Runoff	8651*	2003	New York	Skunk genotype & *C. ubiquitum*	XIIb, XIId	Jiang et al., 2005
Runoff	8514*	2003	New York	Skunk genotype & *C. ubiquitum*	XIIb, XIId	Jiang et al., 2005
Runoff	6858	2002	New York	Skunk genotype & *C. ubiquitum*	XIIb	Jiang et al., 2005

*Multiple PCR products from the storm runoff sample produced different subtypes.

**Letters “a” and “b” at the end of subtype names are used to distinguish subtypes that have the same number of trinucleotide repeats but nucleotide substitutions in the downstream non-repeat region of the gp60 gene.

Table 2

Nucleotide sequence similarity (lower triangular matrix) and potential genetic recombination events (upper triangular matrix) among subtype families of *Cryptosporidium* skunk genotype (XVIa, XVIb, XVIc, and XVId) and chipmunk genotype I (XIVa) at the gp60 locus.

	XVIa	XVIb	XVIc	XVId
XVIa	-	0	0	0
XVIb	77.3%	-	0	1
XVIc	75.9%	76.7%	-	0
XVId	72.1%	73.2%	83.2%	-
XIVa	72.0%	69.6%	66.5%	66.7%