Is Cryptosporidium from the common wombat (Vombatus ursinus) a new species distinct from Cryptosporidium ubiquitum?

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ABSTRACT

The emerging zoonotic pathogen Cryptosporidium ubiquitum has been found in a variety of mammalian hosts, including humans, throughout the world. Advances in the molecular characterization of this parasite using the sequence of the 60 kDa glycoprotein (gp60) gene have allowed the classification of “subtypes”. Sequences derived from faecal samples from the common wombat (Vombatus ursinus) have identified a novel gp60 subtype designated here as C. ubiquitum XIIg. Phylogenetic analysis suggests that subtypes of C. ubiquitum can be divided into generalist and specialist groups, which is important when considering the zoonotic potential of C. ubiquitum in the context of drinking water safety.

Keywords: Cryptosporidium ubiquitum; Vombatus ursinus; common wombat; 60 kD glycoprotein (gp60); ribosomal RNA small subunit (SSU); phylogeny.

Abbreviations:

60 kD glycoprotein (gp60)
ribosomal RNA small subunit (SSU)
Cryptosporidium oocyst wall protein (cowp)

1. Introduction

Currently, 31 species and more than 40 genotypes of Cryptosporidium have been recorded (Holubová et al., 2016; Kváč et al., 2016; Zahedi et al., 2016), of which C. hominis and C. parvum are responsible for the majority of cryptosporidiosis cases in humans (Ryan et al., 2014). The recent recognition of C. ubiquitum as an emerging zoonotic pathogen has elevated concerns within the public health community due to
its apparently ubiquitous occurrence in an array of mammalian hosts throughout the world (Li et al., 2014). Knowledge of the species and/or genotypes present in natural drinking water catchments is crucial for assessing the possible risk of cryptosporidiosis transmission and for developing management strategies (Nolan et al., 2013; Zahedi et al., 2016).

*Cryptosporidium ubiquitum* was formerly called *Cryptosporidium* sp. cervine genotype, until it was officially recognised as a species based on morphological and molecular features (Fayer et al., 2010). Originally, the small subunit of the nuclear ribosomal RNA gene (*SSU*) as well as the *actin* and *Cryptosporidium* oocyst wall protein (*cowp*) genes were employed as markers to identify *C. ubiquitum* by PCR-based tools (reviewed by Fayer et al., 2010). Subsequently, part of the 60 kDa glycoprotein (*gp60*) gene has been used for the classification of subtypes of *C. ubiquitum* (see Li et al., 2014). Based on the sequence of the latter gene, *C. ubiquitum* has been divided into six subtypes: XIIa, Old and New World ruminants; XIIb-XIIId, various New World rodents; XIIe and XIIIf, field mice from the Slovak Republic (Li et al., 2014; Mi et al., 2014; Wang et al., 2014; Guo et al., 2015; Stenger et al., 2015; Qi et al., 2015). Additionally, all subtypes, except XIIe and XIIIf, have been recorded from humans.

*Cryptosporidium* rarely occurs in wombats. To date, there has been one report of *Cryptosporidium* from the common wombat (*Vombatus ursinus*) detected using an immunomagnetic separation/flow cytometry (IMS/FC) technique (Power, 2002, 2010), and another utilizing PCR-based methods (Koehler et al., 2016). In the past six years of monitoring animals in Melbourne’s natural water catchments, 616 faecal samples from wombats were screened using molecular tools for the presence of *Cryptosporidium*; nine of them were test-positive for *SSU*, seven were test-positive
for *C. fayeri* and two were inferred to contain *C. ubiquitum* (see Nolan et al., 2013; Koehler et al., 2016). Here, we used SSU, *gp60* and *actin* gene regions to further molecularly characterize *C. ubiquitum* from faecal deposits from common wombats.

### 2. Materials and methods

The SSU sequences representing *C. ubiquitum* (GenBank accession nos. KU531665 [546 bp] and KU531681 [245 bp]) derived by PCR-based sequencing (Koehler et al., 2016) from the two faecal DNA samples (C3604 and OS5267) from common wombats were available for the present study. Sample C3604 originated from the Cardinia water reservoir catchment (37°47’S 145°24’E; 31 July 2013), and sample OS5267 represented the O’Sullivanassay catchment (37°40’S 145°48’E; 26 November 2014). Sequences KU531665 and KU531681 were identical over 245 bp, and we elected to use the longer (former) sequence for analyses in the present investigation. Here, we sequenced regions of the *gp60* (859 bp) and *actin* (686 bp) genes of *Cryptosporidium* from sample C3604 using an established nested PCR-based approach (cf. Koehler et al., 2016) and deposited them in the GenBank database (accession nos. KX029226 and KX029227, respectively). Then, we used sequence data for all three loci from sample C3604 for phylogenetic analysis using a Bayesian Inference (BI) method, as described previously (Koehler et al., 2016). Other representative sequence data for the SSU (*n* = 35), *gp60* (*n* = 13) and *actin* (*n* = 33) genes were extracted from GenBank (Supplementary Table 1) and used for phylogenetic construction (Figs. 1-3).

### 3. Results and discussion
Koehler et al. (2016) showed that the 245 bp SSU region was identical for *Cryptosporidium* from two faecal samples from wombat (sample C3604 [GenBank accession nos. KU531665] and OS5267 [KU531681]) and that the consensus sequence was consistent with that of *C. ubiquitum* from a goat (accession no. KM199749; 97% similarity); therefore, this particular genotype of *Cryptosporidium*, although rare, has been documented twice in the water catchments (~90 km apart and 482 days apart). We elected to conduct a phylogenetic analysis using 475 bp SSU region representing sample C3604, which revealed a close relationship with four other SSU sequences representing *C. ubiquitum* (96.0 - 98.0 % pairwise similarity; cf. Table 1; Fig. 1) from various host species. However, overall nodal support was weak, resulting in a mostly unsupported polytomy (see Fig. 1). Of note were three sequences that were distinct from the main *C. ubiquitum* polytomy, namely that with accession no. AB697056 representing *C. ubiquitum* from the large Japanese field mouse (*Apodemus speciosus*) from Japan (Murakoshi et al., 2013); that with EF641019 representing *Cryptosporidium* sp. deer mouse genotype IV from a deer mouse (*Peromyscus* sp.) in the USA (Feng et al., 2007); and that with KC962124 representing *C. ubiquitum* from a yellow-necked field mouse (*Apodemus flavicollis*) from Poland (direct submission to GenBank), which had 10-16 nucleotide differences and 96.0-97.5% sequence similarity when compared with a sequence from the common wombat (accession no. KU531665) (Table 1). Upon pairwise comparison of SSU (475 bp), the genetic distances between the wombat sequence and those from other recognised *C. ubiquitum* subtypes were considerably greater than the six nucleotides (98.5% similarity) between the two recognised species, *C. parvum* and *C. hominis*. Although intra-isolate variability of one to two nucleotides is common for the SSU locus (Ryan et al., 2014), greater levels of nucleotide variation have been
recorded (e.g., the name *C. ubiquitum* was retained when there was an 8 bp difference between *C. ubiquitum* from a field mouse compared with the typical genotype for *C. ubiquitum*; see Murakoshi et al., 2013). It is difficult to predict the degree of genetic variability necessary when defining species (Xiao et al., 2004), and the question arises at what point should approximate subtypes be coined/used (e.g., *C. ubiquitum*-like). Consistent with some other species of *Cryptosporidium* (e.g., *C. fayeri, C. macropodum* and *C. ryanae*; Koehler et al., 2016), *C. ubiquitum* is proposed to represent a species complex (cf. cryptic species; Bickford et al., 2007) rather than a unique, relatively homogeneous species based on SSU sequence data. Further work is required to test this hypothesis.

Compared with SSU, the sequences from the actin gene (accession no. KX029227) did not provide additional resolution for differentiating subtypes of *C. ubiquitum* (Fig. 2), as there were only 2-4 nucleotide differences (99.7-99.4% similarity) over 686 bp between the sequences derived from *Cryptosporidium* from wombat and other *C. ubiquitum* representatives. In contrast, the gp60 locus provided more resolution than SSU, enabling the classification of *C. ubiquitum* subtypes (cf. Li et al. 2014). The sequence from *Cryptosporidium* from wombat (KX029226) had most similarity (81.2%, equating to 159 bp over 1049 bp, including indels) with that of *C. ubiquitum* subtype XIIa (Figure 3, Table 2). There were 11 synonymous and 81 non-synonymous substitutions when compared with subtype XIIa. Taken together, the findings of the present molecular characterization of *C. ubiquitum* from the common wombat provide evidence for a unique subtype of *C. ubiquitum* based on the gp60 marker. In accordance with the nomenclature used for naming previous *C. ubiquitum* subtype families (Li et al., 2014), we propose that XIIg be designated as a novel subtype for *C. ubiquitum* found in the common wombat.
Cryptosporidium ubiquitum subtypes XIIe and XIIf from yellow-necked and striped field mice (Apodemus flavicollis and Apodemus agrarius, respectively) sampled from Slovakia form two distinct clades in the phylogenetic tree constructed using gp60 data (Fig. 3). Although respective SSU regions were not sequenced, we suspect that they might relate to the distinctive field mouse samples present in the SSU tree (Fig. 1). If this were the case, there seem to be distinct levels of host specificity/affiliation within what is presently recognized as C. ubiquitum (cf. Li et al., 2014).

Subtypes XIIa to XIIc are known to infect a range of mammals, including humans, ungulates and rodents, and thus represent a group of generalists, whereas subtypes XIIe and XIIf, which are specific to rodents (principally the genus Apodemus) appear to be specialists, like subtype XIIg presently recognised as being specific to wombats. Another perspective is that subtypes XIIe-XIIg, found external to the main clade of C. ubiquitum comprising subtypes XIIa-XIIc (cf. Fig. 1), might be distinct species of Cryptosporidium. This latter proposal would require further morphological and biological investigations (Xiao et al., 2004). We also suggest that a reassessment of the C. ubiquitum complex is needed. If C. ubiquitum XIIg were a novel species, it would be the third marsupial-specific species of Cryptosporidium, in addition to C. macropodum and C. fayeri (see Power 2010; Ryan and Power 2012). The close proximity of C. ubiquitum to C. macropodum within the SSU tree (Fig. 1) might be a signal of a deeper evolutionary tie between C. ubiquitum XIIg and C. macropodum. Clearly, further sampling of marsupials, as well as monotremes, for Cryptosporidium would likely provide a more meaningful insight into the diversity of Cryptosporidium.

In conclusion, we provide molecular evidence for the presence of a novel C. ubiquitum subtype (C. ubiquitum XIIg) from the common wombat in Australia based
on sequences of three independent gene regions (SSU, actin and gp60). Furthermore, the naming of taxa, particularly those regarded as emerging human pathogens, should be scrutinized. Knowledge of the zoonotic potential of each particular Cryptosporidium species and/or subtypes (e.g., generalist strain found in a wide variety of animals and humans versus a specialist strain specific to marsupials) is crucial because of the consequences associated with alerting health authorities to the presence of potentially zoonotic human pathogens. This knowledge is especially relevant when monitoring natural catchments supplying unfiltered drinking water to communities such as those in the city of Melbourne.

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Supplementary Data

Supplementary data related to this article can be found at

http://dx.doi.org/XXXXXXXXXX.
References


Figure Legends

Fig. 1. Phylogenetic relationships of selected Cryptosporidium species inferred from partial sequence (475 bp) of the small subunit rRNA (SSU) gene using Bayesian inference. Posterior probabilities are indicated for select nodes. Cryptosporidium muris was used as an outgroup. GenBank accession numbers precede species names. Cryptosporidium ubiquitum from the common wombat (featured in this study) is in bold-type.

Fig. 2. Phylogenetic relationships of selected Cryptosporidium species inferred from partial sequence (686 bp) of the actin gene using Bayesian inference. Posterior probabilities are indicated for select nodes. Plasmodium falciparum was used as an outgroup. GenBank accession numbers precede species names. Cryptosporidium ubiquitum from the common wombat (characterized in this study) is in bold-type.

Fig. 3. Phylogenetic relationships of selected Cryptosporidium ubiquitum subtypes inferred from partial sequence (~1050 bp) of the 60 kD glycoprotein (gp60) gene using Bayesian inference. Posterior probabilities are indicated for select nodes. Cryptosporidium ubiquitum subtype XIIIf was used as an outgroup. GenBank accession numbers precede species names. Cryptosporidium ubiquitum from the common wombat (characterized in the present study) is in bold-type.
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