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Cryptosporidium viatorum from the native Australian swamp rat *Rattus lutreolus* - An emerging zoonotic pathogen?

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ABSTRACT

Cryptosporidium viatorum is a globally distributed pathogenic species of *Cryptosporidium* that has only ever been recorded from humans, until now. For the first time, we molecularly characterised a novel subtype of *C. viatorum* (subtype XVbA2G1) from the endemic Australian swamp rat (*Rattus lutreolus*) using the small subunit of nuclear ribosomal RNA (*SSU*) gene and then subtyped it using the 60-kilodalton glycoprotein (*gp60*) gene. In total, faecal samples from 21 swamp rats (three were positive for *C. viatorum*), three broad toothed rats (*Mastacomys fuscus*) and two bush rats (*Rattus fuscipes*) were tested for *Cryptosporidium*. The long-term, isolated nature of the swamp rat population in Melbourne's drinking water catchment system (where public access is prohibited), the lack of *C. viatorum* from other mammals and birds living within the vicinity of this system and its genetic distinctiveness in both the *SSU* and *gp60* gene sequences from other species of *Cryptosporidium* collectively suggest that *C. viatorum* might be endemic to native rats in Australia. The current state of knowledge of epidemiological surveys of *Cryptosporidium* of rats and the zoonotic potential are further discussed in light of the finding of *C. viatorum*. Long-term studies, with the capacity to repetitively sample a variety of hosts in multiple localities, in different seasons and years, will allow for greater insight into the epidemiological patterns and zoonotic potential of rare *Cryptosporidium* species such as *C. viatorum*.

1. Introduction

Cryptosporidium is a genus of protozoan parasites that are recognised as a leading cause of diarrhoea and malnutrition, particularly in developing regions around the world (Sow et al., 2016; Kotloff, 2017; Squire and Ryan, 2017). At least 34 species and more than 40 genotypes are recognised to infect humans and other animals (Zahedi et al., 2016), 21 of which are reported to have zoonotic potential (Xiao and Fayer, 2008; Ryan et al., 2014; Zahedi et al., 2016; Xiao and Feng, 2017). *Cryptosporidium viatorum* was first described in 2012 from travellers returning to the United Kingdom from the Indian subcontinent (Elwin et al., 2012). Thus far, *C. viatorum* has been found in people endemic to or returning from the following countries: Bangladesh, Barbados, Colombia, Ethiopia, Guatemala, India, Kenya, Nepal, Nigeria and Pakistan (Elwin et al., 2012; Insulander et al., 2013; Lebbad et al., 2013; Adamu et al., 2014; Ayinmode et al., 2014; Stensvold et al., 2015; de Lucio et al., 2016; Sanchez et al., 2017; Ukwah et al., 2017).

Clinical symptoms associated with cryptosporidiosis linked to *C. viatorum* from Swedish and British-based travellers to Bangladesh, Guatemala, India, Kenya, Nepal and Pakistan have included diarrhoea, abdominal pain, nausea, fever, headache, vomiting and marked weight loss, with illness lasting from 9 to 30 days (Elwin et al., 2012; Lebbad et al., 2013). Other studies reporting *C. viatorum* infection were in HIV-positive patients or children; the symptoms could either not be distinguished or were not recorded (Adamu et al., 2014; Ayinmode et al., 2014; de Lucio et al., 2016; Sanchez et al., 2017; Ukwah et al., 2017).

Because *C. viatorum* is currently the only species of *Cryptosporidium* found exclusively in humans, there has been speculation as to whether *C. viatorum* occurs in a domestic or sylvatic animal reservoir host (Elwin et al., 2012; Lebbad et al., 2013; Stensvold et al., 2015; Sanchez et al., 2017). However, to date, there has been no report of *C. viatorum* in an animal species other than human.

The Melbourne Water project (Nolan et al., 2013; Koehler et al., 2016b) is an ongoing survey of eukaryotic microbes including *Cryptosporodium* and *Giardia* (since June of 2009) in faecal deposits from feral and endemic wildlife within the closed water catchments supplying the city of Melbourne, Australia, with drinking water. Of the faecal samples collected and tested to date, the majority has been from

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eastern grey kangaroos, European rabbits, Sambar deer, swamp wallabies and common wombats. Interest in pathogens of lesser studied species of native wildlife led to the collection of faeces from three of Australia's native rat species, the Australian swamp rat, *Rattus lutreolus*, the bush rat, *Rattus fuscipes* and the broad-toothed rat, *Mastacomys fuscus*.

Here, we molecularly characterise a novel subtype of *Cryptosporidium* from a native rat and compare it to existing subtypes of *C. viatorum* using markers from the small subunit of nuclear ribosomal RNA (*SSU*) gene and the 60 kilodalton glycoprotein (*gp*60) gene which allowed for the subtyping of *C. viatorum* (Stensvold et al., 2015). We also appraise the survey history of *Cryptosporidium* from all known species of rats.

2. Materials and methods

2.1. Sample collection and DNA extraction

Since June of 2009, the flood plain where the Yarra River feeds the Upper Yarra Reservoir (~100 km east of Melbourne [latitude: −37.673563; longitude: 145.89612]) was surveyed 17 times as part of our ongoing Melbourne Water project which monitors wildlife faecal samples from multiple catchments on a monthly basis for potential waterborne pathogens (Nolan et al., 2013; Koehler et al., 2016b). A total of 1394 faecal samples from foxes, cats, deer, kangaroos, rabbits, rats, wombats and waterbirds (mostly Australian wood ducks) have been collected from within the 'closed' catchment called Upper Yarra. A closed catchment refers to the land surrounding a reservoir where public access is prohibited and human activity is restricted to employees of the water management corporation. The Upper Yarra catchment (33,670 hectares) was established in 1888, in accordance with the Closed Catchment Policy that set aside the land for drinking water collection (Parks Victoria, 2002). *Cryptosporidium* species and genotypes that have been discovered in the Upper Yarra catchment include: *C. cuniculus* (from rabbits), *C. hominis*, *C. parvum*, *C. ryanae*, *C. ubiquitum* (from deer) and *Cryptosporidium* sp. duck-like genotype (from waterbirds) (Nolan et al., 2013; Koehler et al., 2016b).

In recent years, with the growth of grasses and reeds along the banks of the reservoir, we have noticed well-defined rodent runways (Koehler and Haydon, personal observations). Rat faeces were collected on three occasions: 3 September, 2015 (n = 12 samples) (Spring), 27 July, 2016 (n = 1) (Winter) and 26 April, 2017 (n = 13) (Autumn). Faecal samples were identified in the field as belonging to rodents based on the identification of rodent runways and faecal morphology (Triggs, 2004). Samples were taken in a 'haphazard' collection manner (Manly and Navarro Alberto, 2015), while trying to avoid collecting faeces from the same runway to minimise duplication of collection from the same host. DNAs were extracted from faecal samples using the MoBio (Carlsbad, CA, USA) described previously (Koehler et al., 2016b).

2.2. Host identification

The molecular identification of the rodent hosts using faecal DNA was achieved by PCR-based amplification of a 421 bp region employing universal vertebrate cytochrome *b* (*cyt*b) primers mcb398 5′-TAC CAT GAG GAC AAA TAT CAT TCT G-3′ and mcb869 5′-CCT CCT AGT TTG TTA GGG ATT GAT CG-3' (Verma and Singh, 2003) and analysis of amplicons.

PCR was carried out in a reaction volume of 50 µl using a standard reaction buffer, 3.0 mM of $MgCl_2$, 200 µM of each dNTP, 50 pmol of each primer, 1 U of Go*Taq* polymerase (Promega, USA) and 2 µl of genomic DNA (except for the no-template controls, where H_2O was added). The PCR conditions were: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 s (denaturation), 51 °C for 30 s (annealing) and 72 °C for 30 s (extension), with a final extension of 72 °C for 5 min. A restriction fragment length polymorphism (RFLP) analysis was conducted using the FastDigest *Taq*I enzyme (Thermo Fisher Scientific, USA) according to the manufacturer's protocol; digests were separated in a 1.5% agarose gel by electrophoresis for 30 min at 90 V. Amplicons representing each unique banding patterns were selected, individually treated with shrimp alkaline phosphatase and exonuclease I (Thermo Fisher Scientific), according to the manufacturer's instructions, and then subjected to bi-directional automated sequencing (BigDye® Terminator v.3.1 chemistry, Applied Biosystems, USA) using the same primers as for PCR amplification. The resultant sequences were compared with sequences in GenBank using the BLASTn algorithm, and levels of identity established. Sequences from this study were deposited in the GenBank database under the accession numbers MG021319 – MG021323.

Host identification was achieved by phylogenetic analyses of the *cyt*b sequence data, conducted by the neighbor joining (NJ) distance method (Saitou and Nei, 1987) in the program MEGA v.7.0.20 (Kumar et al., 2016). Evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000), including transitions and transversions for the nucleotide data. Rates of evolution among sites were considered uniform and gaps were treated using pairwise deletion. A total of 2000 bootstrap replicates were performed and are reported as bootstrap percentages (BP).

2.3. Literature survey of Cryptosporidium in rats

A comprehensive literature search was performed using Google Scholar (google.scholar.com) using search terms "rat", "*Rattus*" and "*Cryptosporidium*" in order to find all known epidemiological surveys of rats for *Cryptosporidium*. The search results, including host, locality, prevalence and parasite species or genotype, are summarised in Supplementary Table 1.

2.4. Nested PCR assays for Cryptosporidium

Nested PCR assays were carried out for the small subunit of nuclear ribosomal RNA (*SSU*) (Alves et al., 2003) and 60-kilodalton glycoprotein (*gp*60) (Stensvold et al., 2015) genes using the same set-up as described in section 2.2, except that for the secondary PCR, 1 µl of template from the primary PCR was carried over to the secondary PCR. No-template (negative) controls were included at all steps, and no-template controls were carried over from the primary to the secondary (nested) PCR. A well-known positive control sample (*C. parvum* DNA) was included in each PCR run.

An established *SSU*-nested PCR was conducted as described previously (Alves et al., 2003). In brief, the primary PCR (~760 bp) was carried out using primers 18SiCF2 (forward: 5′-GAC ATA TCA TTC AAG TTT CTG ACC-3′) and 18SiCR2 (reverse: 5′-CTG AAG GAG TAA GGA ACA ACC-3′), followed by secondary (nested) PCR (~590 bp) using primers 18SiCF1 (forward: 5′-CCT ATC AGC TTT AGA CGG TAG G-3′) and 18SiCR1 (reverse: 5′-TCT AAG AAT TTC ACC TCT GAC TG-3′). Both the primary and secondary PCRs utilized the following cycling conditions: 94 °C for 5 min (initial denaturation), followed by 45 cycles of 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing) and 72 °C for 30 s (extension), with a final extension of 72 °C for 10 min.

Once it was determined that the taxon of *Cryptosporidium* identified was related to *C. viatorum,* the nested PCR protocol for *gp*60 of *C. viatorum* was conducted essentially as described by Stensvold et al. (2015). In brief, primary PCR of a partial region (1192 bp) of the *gp*60 gene from 2 µl of genomic DNA (expect for no-template controls) was conducted using primers CviatF2 (forward 5′-TTC ATT CTG ACC CCT CAT AG-3′) and CviatR5 (reverse: 5′-GTC TCC TGA ATC TCT GCT TAC TC-3′); 1 µl of template from the primary PCR was carried over to the secondary PCR conducted using primers CviatF3 (forward: 5′-GAG ATT GTC ACT CAT CAT CGT AC-3′) and CviatR8 (reverse: 5' –CTA CAC GTA AAA TAA TTC GCG AC-3′) to produce a product of ~950 bp. Both