

# Comparative genetic diversity of *Cryptosporidium* species causing human infections

## Research Article

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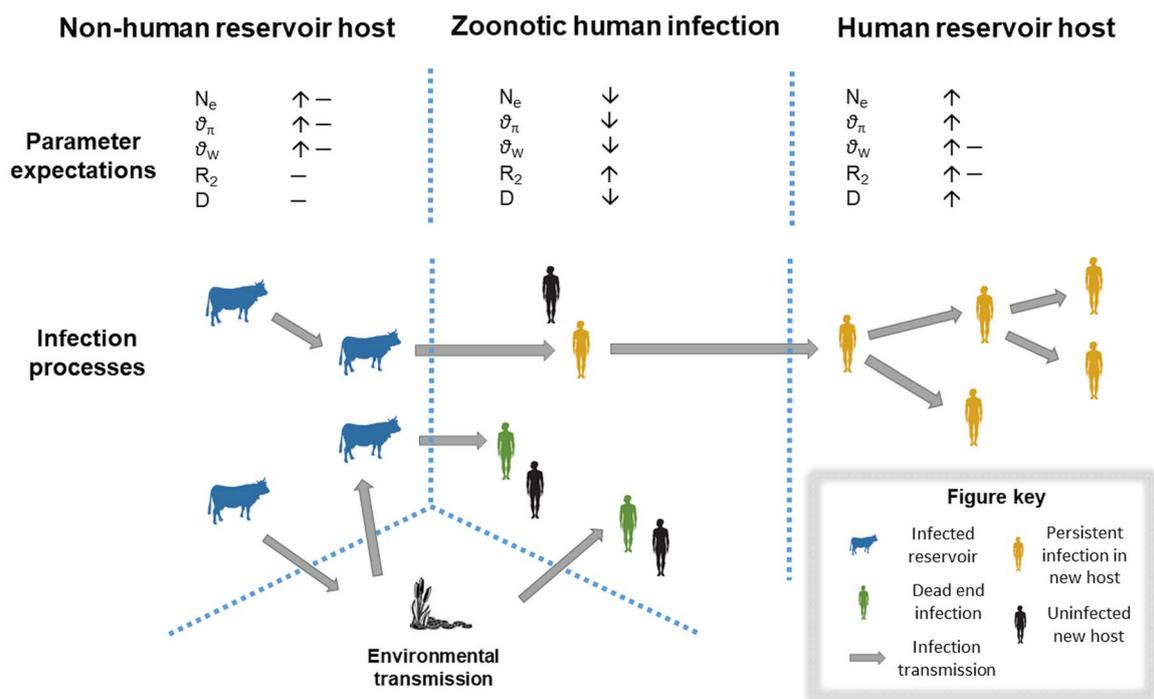
### Abstract

Parasites sometimes expand their host range and cause new disease aetiologies. Genetic changes can then occur due to host-specific adaptive alterations, particularly when parasites cross between evolutionarily distant hosts. Characterizing genetic variation in *Cryptosporidium* from humans and other animals may have important implications for understanding disease dynamics and transmission. We analyse sequences from four loci (gp60, HSP-70, COWP and actin) representing multiple *Cryptosporidium* species reported in humans. We predicted low genetic diversity in species that present unusual human infections due to founder events and bottlenecks. High genetic diversity was observed in isolates from humans of *Cryptosporidium meleagridis*, *Cryptosporidium cuniculus*, *Cryptosporidium hominis* and *Cryptosporidium parvum*. A deviation of expected values of neutrality using Tajima's *D* was observed in *C. cuniculus* and *C. meleagridis*. The high genetic diversity in *C. meleagridis* and *C. cuniculus* did not match our expectations but deviations from neutrality indicate a recent decrease in genetic variability through a population bottleneck after an expansion event. *Cryptosporidium hominis* was also found with a significant Tajima's *D* positive value likely caused by recent population expansion of unusual genotypes in humans. These insights indicate that changes in genetic diversity can help us to understand host-parasite adaptation and evolution.

## Introduction

Understanding the complex dynamics of host shifting in parasites is vital for the effective development of controls to prevent their transmission to people. Cross-species transmission can be influenced by rates of host–parasite contact, host immune response and parasite adaptation (Lloyd-Smith *et al.*, 2009). For many microorganisms, genetic adaptation is often proposed to be the key driver of successful emergence into a new host species, but it is difficult to distinguish which processes generate genetic change (Pepin *et al.*, 2010; Chabas *et al.*, 2018). The genetic variability of parasites in new hosts may provide some insights into the evolutionary processes driving adaptation (Penczykowski *et al.*, 2016). Comparative analysis of parasite genetic diversity within and among hosts could help to clarify transmission–evolution dynamics, as well as their associated risk for host shifts and outbreaks among people. Distinguishing changes in genetic patterns is fundamental for monitoring pathogen evolution, adaptation to new hosts, identification of factors driving cross-species transmission and, ultimately, the reduction of risk to public health.

*Cryptosporidium* is one of the most common enteric parasites in humans. This parasite causes infant deaths from diarrhoea, as well as disease in wildlife and domestic animals (Kotloff *et al.*, 2013; Striepen, 2013). Most of the approximately 38 species of *Cryptosporidium* and several related genotypes are adapted to different vertebrate hosts, which limits between-species transmission (Xiao and Feng, 2008; Garcia-R and Hayman, 2016, 2017; Garcia-R *et al.*, 2017; Feng *et al.*, 2018). Humans are the major host of *Cryptosporidium hominis* and *Cryptosporidium viatorum* (Ryan *et al.*, 2014), but *C. hominis* is reported in other mammal species (Widmer *et al.*, 2020), whereas no alternative animal reservoir has been identified for *C. viatorum* (Elwin *et al.*, 2012; but see Koehler *et al.*, 2018). Nevertheless, about 17 *Cryptosporidium* species with diverse animals as main hosts are now found in humans at different notification levels. For instance, cattle are an important reservoir of *C. parvum*, but this species is reported in a significant proportion of human *Cryptosporidium* infections (Feng *et al.*, 2018). Likewise, *Cryptosporidium meleagridis*, *C. cuniculus*, *C. tyzzeri* and *C. ubiquitum* (previously known as the cervine genotype) primarily parasitize birds, rabbits, rodents and ruminants, respectively. These species have recently been found in gastroenteritis cases of humans, but are less commonly reported (Chalmers *et al.*, 2009a; Bouzid *et al.*, 2013; Rašková *et al.*, 2013; Li *et al.*, 2014). This apparent expansion of host range demonstrates the plasticity of these species and their ability to adapt to new niches.



**Fig. 1.** Population and genetic parameter expectations during *Cryptosporidium* host shifting. Key transmission pathways show non-human reservoirs (blue) infecting each other and the environment through oocyst shedding. Cross-species transmission and host shifting occur through direct and indirect transmission (e.g. via the environment), mostly leading to dead-end infections (green). Subsequent establishment in new hosts with possible adaptation through ongoing transmission may occur (yellow). Transmission between individuals of the new host is necessary to establish long-term associations that characterize a successful host shift, in contrast to occasional spillover pathogen infections in the new host. The expected ranges (e.g. ↑ = high, ↓ = low, - = no change, ↑ - = high or no change) for population genetic parameters are shown above each scenario;  $N_e$  – effective population size;  $\theta_\pi$  – nucleotide diversity;  $\theta_W$  – Watterson’s theta;  $R_2$  – Ramos-Onsins and Rozas’ population growth test; and  $D$  – Tajima’s  $D$ .

The change to new environments (here, new hosts) must be associated with traits that influence parasite fitness and hence cause signatures in the genetic diversity of the parasites (Barrett *et al.*, 2008). Changes in parasite gene frequencies can be estimated by comparison of genetic variation in the known reservoir host source (where the infection is maintained and perpetuates) and the new host (Fig. 1). The comparison of quantitative genetic differences can help to untangle the processes involved in parasite–host dynamics, and the rates and barriers parasites need to overcome to enable successful cross-species transmissions (Geoghegan *et al.*, 2017). While low genetic diversity in the parasite that colonizes a host does not necessarily imply that an episode of shifting has occurred, it is strongly indicative of genetic factors affecting adaptation and highlights the importance of comparative studies across multiple populations and species (Fig. 1).

*Cryptosporidium* species in a large, well-mixed host population might have high genetic diversity, with their transmission dynamics stable over time (Fig. 1). However, during a parasite population expansion and founder event, a large chain of cascading effects may influence these parameters (Longdon *et al.*, 2014). Infectious doses for humans can be as few as 10 infectious oocysts, each with four diploid sporozoites within the oocysts. Such population bottlenecks typically promote loss of genetic diversity in the parasite population of the new host and generate among-population genetic differences. Upon infection, however, populations may expand massively, with infected hosts excreting up to  $6 \times 10^7$  oocysts per gram of feces (Uga *et al.*, 2000) and up to  $10^{10}$  oocysts per day for up to 2 weeks while infected (Meinhardt *et al.*, 1996). The parasite population will reach a recovery point after the colonization event if the host population is large and interconnected (Fig. 1). Although host species differ in many traits, including population size, distribution and

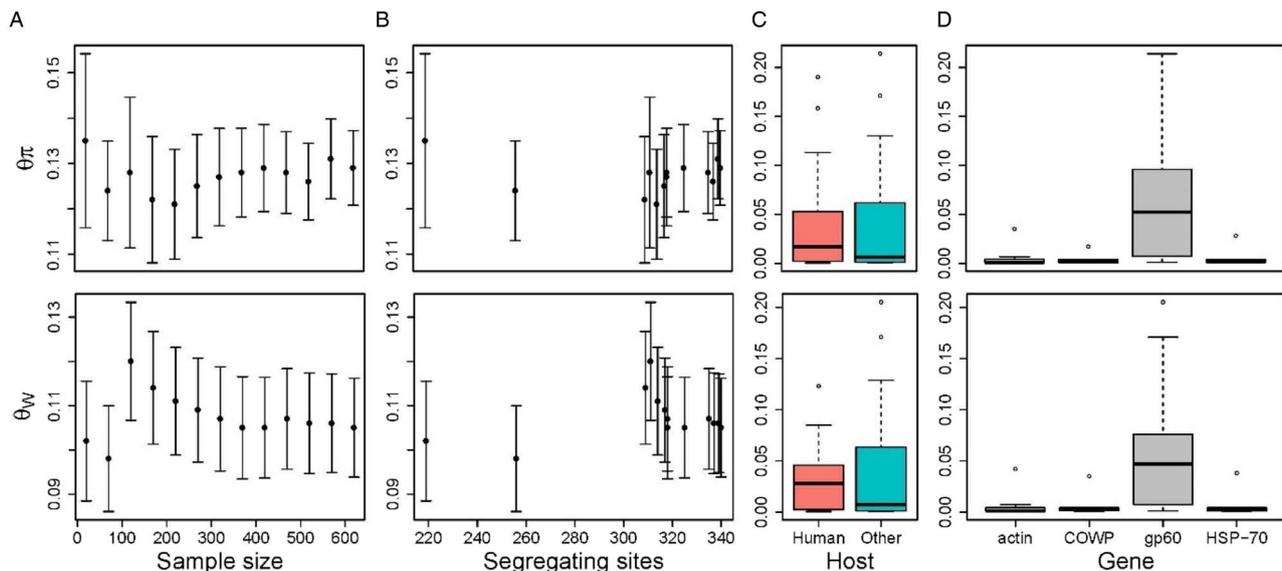
resistance, we predict that there is a relationship between the dynamics of host jumps and genetic changes in parasite populations of *Cryptosporidium* that records important clues to their transmission into new hosts.

Here, we use a population-based study to infer patterns of genetic diversity from host shifting in *Cryptosporidium* parasites. We study *Cryptosporidium* genetic variation across host species using publicly available data to discern different likely outcomes of host–parasite dynamics and the cause-effect relationship of *Cryptosporidium* parasites that host shift. Our aim is to help understand host–parasite shifts, such as for human cryptosporidiosis, by addressing the following questions: can general patterns of changes in genetic diversity be detected in the genes of parasites that host switch? Are genetic diversity measures lower in *Cryptosporidium* species recently reported in humans? How is the genetic diversity of *Cryptosporidium* species distributed across different animal hosts?

## Methods

### Data collection

We use data from previously published sequences of *Cryptosporidium* species found in GenBank (see Table S1 for details). Here, we defined populations and genetic diversity as genetic variants on six evolutionary lineages of *Cryptosporidium* (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. cuniculus*, *C. tyzzeri* and *C. ubiquitum*) detected in both humans and other vertebrates around the world. Nucleotide sequences of interest were downloaded using Geneious v.10.2 (Kearse *et al.*, 2012) with a search strategy comprised of fields including species names [‘Organism’] and gene names [‘All Fields’ or ‘Protein Name’]. The 60-kDa glycoprotein (gp60), heat shock protein (HSP-70),



**Fig. 2.** Rarefaction analysis of the stability of genetic diversity measures ( $\theta_{\pi}$  and  $\theta_w$ ) from publicly available data in *Cryptosporidium*. Outputs derived as a function of downsampling individuals (A) or segregating sites (B) in a number of gp60 sequences. Nucleotide diversity ( $\theta_{\pi}$ ) and Watterson's estimator ( $\theta_w$ ), upper and lower respectively, by host (C) and genes (D).

oocyst wall protein (COWP) and actin gene (actin) are the most frequent markers used for detection and identification of *Cryptosporidium*, and we, therefore, focus on these four genes here. Among these markers, gp60 is the only gene considered to be under selection (Widmer, 2009; Abal-Fabeiro *et al.*, 2013) and its adaptive evolution plays a powerful role in pathogen entrenchment across species boundaries. This marker has, however, been readily and extensively adopted as a key component for molecular epidemiological investigations because of its high reliability in the characterization of genotypes and detection of variants within populations (Garcia-R and Hayman, 2017; Garcia-R *et al.*, 2020).

Very short sequences (<200 bp) or sequences overlapping only a short section of the overall alignment were discarded (see Supporting Information). Related meta-information (host and isolation/source) was appended to the final dataset and sequences were organized by species, gene, host and source (feces-only to ensure that samples were obtained only from the specific host). Hosts were chosen according to common names when the scientific name was not available and when the names indicate that sequences are from the given host species. In population genetic studies, assigning individuals to populations may be challenging if there is incomplete knowledge of the sampling design. Due to the low number of samples from geographically and taxonomically distant hosts, we compared the data obtained from humans to a combined dataset of all non-human hosts. The reason for this approach is to test for evidence of diversity estimates supporting our expectations (Fig. 1).

### Analyses

Nucleotide diversity ( $\theta_{\pi}$ ) and Watterson's theta ( $\theta_w$ ) within hosts were estimated for sequences of *Cryptosporidium* species associated with humans and other hosts using DnaSP v.5.0 (Librado and Rozas, 2009). The Tajima's *D* statistic (Tajima, 1989) was used to test the null hypothesis of neutrality and demographic growth was assessed using the *R*<sub>2</sub> test (Ramos-Onsins and Rozas, 2002), both using DnaSP v.5.0. Sensitivity analyses were performed to test the effect of sample size and the number of segregating sites (Subramanian, 2016). We advocate for subsampling the data to ascertain the robustness of the summary statistics

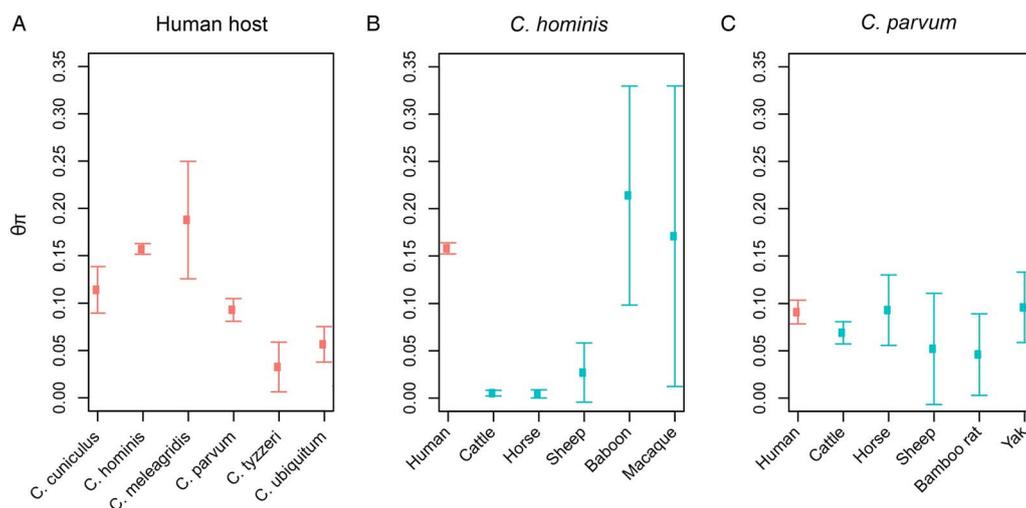
using a rarefaction approach for our most data-rich sample, the gp60 gene, which displays a high mutation rate and high level of polymorphisms (Strong *et al.*, 2000).

### Results

Sequences used in this study were submitted to GenBank between 2002 and 2017, with the exception of a few COWP gene sequences isolated from humans in previous years. This is important due to nomenclature changes in *Cryptosporidium*. For instance, *C. hominis* was referred to as *C. parvum* genotypes H or I before 2002, which can create confusion when assigning sequences to species and influence the results of genetic diversity analyses. In total, we compiled 697 sequences of the gp60 gene from *C. cuniculus*, *C. hominis*, *C. meleagridis*, *C. parvum*, *C. tyzzeri* and *C. ubiquitum* that infect humans, as well as isolates from a range of different hosts (462 sequences from humans and 235 sequences from other animals); 13 sequences of the HSP-70 gene from *C. hominis* (11 sequences from humans and two sequences from other animals); eight sequences of the actin gene from *C. parvum* (five sequences from humans and three sequences from other animals); and 46 sequences of the COWP gene from *C. parvum* (18 sequences from humans and 28 sequences from other animals) (Table S1).

Genetic diversity estimates, including standard deviations, are provided in Table S2. We performed sensitivity analyses and did not find a significant difference between data partitions as a result of reductions in either sample size or the number of segregating sites on  $\theta_{\pi}$  and  $\theta_w$  (Fig. 2A and B), showing that the estimates of these summaries are robust across a range of sample sizes in this instance. We confirmed that gp60 has the highest variation, but that genetic diversity estimates are not statistically significant among other genes and hosts (Fig. 2C and D).

Population parameter estimates yield a high  $\theta_{\pi}$  for the gp60 gene in humans infected with *C. meleagridis* and *C. cuniculus* in comparison to their other hosts (Fig. 3A and Table S2). In contrast, *C. hominis* presents high  $\theta_{\pi}$  values in its major host group (humans and non-human primates; Fig. 3B) suggesting a bottleneck and likely a dead-end infection in domesticated animals. The more ubiquitous *C. parvum* has low values in its major host source (cattle) but similar values across several other hosts, even



**Fig. 3.** Population estimates of *Cryptosporidium* species infecting humans and other hosts. (A) Estimates of nucleotide diversity ( $\theta_{\pi}$ ) using the gp60 gene for six *Cryptosporidium* species infecting humans (red); and variation in nucleotide diversity ( $\theta_{\pi}$ ) in gp60 in *C. hominis* (B) and *C. parvum* (C) among other hosts (blue).

**Table 1.** Estimates of nucleotide diversity ( $\theta_{\pi}$ ), Watterson's estimator ( $\theta_W$ ), Tajima's  $D$  and Ramos-Onsins and Rozas' population growth test ( $R_2$ ) using gp60 and COWP genes for *Cryptosporidium* species infecting humans and other animal hosts ( $n \geq 4$ , the minimum number of sequences required to compute these tests). \* $P < 0.05$ , \*\*\* $P < 0.001$ .

Gene	Species	Host	$\theta_{\pi}$	S.D.	$\theta_W$	S.D.	$D$	$R_2$
gp60	<i>C. cuniculus</i>	Others	0.081	0.033	0.076	0.006	0.33	0.19
		Humans	0.113	0.013	0.065	0.005	<b>2.99***</b>	0.24
	<i>C. hominis</i>	Others	0.1405	0.033	0.16	0.013	-0.48	0.11
		Humans	0.158	0.003	0.084	0.006	<b>2.76*</b>	0.16
	<i>C. meleagridis</i>	Others	0.075	0.015	0.067	0.006	0.58	0.21
		Humans	0.19	0.033	0.123	0.011	<b>2.23*</b>	0.21
	<i>C. parvum</i>	Others	0.073	0.005	0.054	0.004	1.13	0.12
		Humans	0.091	0.0064	0.085	0.0046	0.26	0.09
<i>C. ubiquitum</i>	Others	0.099	0.018	0.09	0.006	0.56	0.18	
	Humans	0.053	0.01	0.046	0.0045	0.70	0.19	
COWP	<i>C. parvum</i>	Others	0.0011	0.0008	0.00414	0.0015	-1.15	0.19
		Humans	0.017	0.0081	0.035	0.0045	<b>-2.08*</b>	0.16

though they cover greater taxonomic distances (Fig. 3C). The intermediate levels across the board of hosts might suggest a sustained infection across a broad host range. The same pattern was found in *C. meleagridis* and *C. tyzzeri* with their likely host groups (birds and rodents, respectively) presenting low  $\theta_{\pi}$  values (Table S2). Evidence of distortion in the frequency of polymorphism distributions (Tajima's  $D$ ) was found in two species of *Cryptosporidium* that have been recently reported in humans (*C. meleagridis* and *C. cuniculus*), as well as the type human species (*C. hominis*), at the gp60 gene (Table 1).

## Discussion

Understanding the factors that influence the success of *Cryptosporidium* to colonize new hosts can help to explain host shifting in humans. The putative main hosts of *C. meleagridis* (birds), *C. cuniculus* (rabbits) and *C. parvum* (cattle) were found to harbour low genetic diversity at the gp60 gene compared to the diversity found in humans. Greater  $\theta_{\pi}$  of these parasites in humans did not meet our expectations of high diversity within major hosts and low diversity in new ones (here, humans;

Fig. 1). Nonetheless, significant Tajima's  $D$  positive values in *C. meleagridis* and *C. cuniculus* in humans at the gp60 gene might indicate that these lineages have recently decreased their level of genetic variability through a population bottleneck caused by recent colonization of a new host. These species have lately expanded their range to humans and caused outbreaks worldwide (Chalmers *et al.*, 2009b, 2011; Koehler *et al.*, 2014). An increased number of observed polymorphisms at the gp60 gene must be the result of advantageous adaptations during colonization of the new host and subsequent 'arms race', indicating that these parasites are either human-adapted or often cause infection in people (Akiyoshi *et al.*, 2003; Feng *et al.*, 2018).

The genetic diversity of *C. hominis* observed in people matches our expectations for the reservoir host-parasite relationship. There are genetically diverse *C. hominis* genotypes identified in humans causing unusual infections (Lebbad *et al.*, 2013, 2018), as well as in other hosts (Plutzer and Karanis, 2009; Feng *et al.*, 2018). This might indicate a predictable relationship between genetic diversity and taxonomic distance of primary and secondary hosts (i.e. similar parasite diversity in closely related hosts). High variable genotypes lead to an increase in genetic

polymorphisms that cause a deviation from the theoretical expectation of neutrality. Differences in genetic diversity could be due to higher rates of adaptation in lineages with large population sizes (Feng *et al.*, 2018) that allow the parasite to respond to novel selection pressures from the host(s).

Despite the high genetic diversity in *Cryptosporidium* species that infect humans, the range of potential evolutionary processes in host–parasite interactions needs to be better understood (Barrett *et al.*, 2008; Longdon *et al.*, 2014). We aim to draw inferences about patterns of host–parasite associations and evolutionary history of episodic host shifting or adaptation within this system (Fig. 1). Nonetheless, we acknowledge that information on the timing of shifting or details of the cross-species transmission events is currently limited. The precise effects of host shifting in *Cryptosporidium* populations could depend on many factors including the life cycle, population subdivision, sampling strategy (markers under selection pressure or mixed geographical origin of the samples), reproductive mode, genetic recombination, host phylogeny and host resistance to parasites (Lively, 2010; Wang *et al.*, 2014), among others.

Sampling strategies play a key role in observed population summary statistics and we need to study the actual evolutionary units in time and space. Unfortunately, publicly available data are invariably found in scattered locations due to piecemeal submission of sequences to genetic databases and different questions and research designs from the original studies. The scale at which *Cryptosporidium* species adapt to new hosts might depend on the properties and dynamics of the given system. For instance, a species with a time-dependent association for host adaptation and large geographic and host ranges may be more likely to adapt through multiple, geographically-restricted mutations than by global sweeps of local and host-restricted species (Leffler *et al.*, 2012). Studies at multiple scales are required to tease these processes apart, but clearly defining and delimiting the population being studied is essential for accurate estimates of host shifting.

We previously showed that departures from neutrality in *Cryptosporidium* from humans in New Zealand could be detected through changing epidemiological patterns (Garcia-R and Hayman, 2017). This means that analysing disease patterns by fine-scale surveillance is likely a more powerful way to infer parasite dynamics. With the advent of cheap and rapid sequencing of whole genomes, we expect that understanding genetic diversity and population structure will provide explanatory power to identify the risk of the spread of *Cryptosporidium* species and ecological fitting into humans (Nader *et al.*, 2019). Ideally, studies using genomes and fine-scale longitudinal sampling before and after the putative host shifting event will help to identify the patterns in genetic diversity variation caused by parasite evolutionary processes, which can, in turn, be used to predict novel host shifts from genetic data alone.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182020001493>

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**Conflict of interest.** The authors declare that there is no conflict of interest.

**Ethical standards.** Not Applicable.

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