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Disentangling social, environmental, and zoonotic transmission pathways of a gastrointestinal protozoan (*Blastocystis* spp.) in northeast Madagascar

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Abstract

Objectives: Understanding disease transmission is a fundamental challenge in ecology. We used transmission potential networks to investigate whether a gastrointestinal protozoan (*Blastocystis* spp.) is spread through social, environmental, and/or zoonotic pathways in rural northeast Madagascar.

Materials and Methods: We obtained survey data, household GPS coordinates, and fecal samples from 804 participants. Surveys inquired about social contacts, agricultural activity, and sociodemographic characteristics. Fecal samples were screened for *Blastocystis* using DNA metabarcoding. We also tested 133 domesticated animals for *Blastocystis*. We used network autocorrelation models and permutation tests (network *k*-test) to determine whether networks reflecting different transmission pathways predicted infection.

Results: We identified six distinct *Blastocystis* subtypes among study participants and their domesticated animals. Among the 804 human participants, 74% ($n = 598$) were positive for at least one *Blastocystis* subtype. Close proximity to infected households was the most informative predictor of infection with any subtype (model averaged OR [95% CI]: 1.56 [1.33–1.82]), and spending free time with infected participants was not an informative predictor of infection (model averaged OR [95% CI]: 0.95

[0.82–1.10]). No human participant was infected with the same subtype as the domesticated animals they owned.

Discussion: Our findings suggest that *Blastocystis* is most likely spread through environmental pathways within villages, rather than through social or animal contact. The most likely mechanisms involve fecal contamination of the environment by infected individuals or shared food and water sources. These findings shed new light on human-pathogen ecology and mechanisms for reducing disease transmission in rural, low-income settings.

KEYWORDS

Blastocystis, infectious disease transmission, Madagascar, network analysis

1 | INTRODUCTION

Understanding the transmission pathways that facilitate the spread of microorganisms among hosts is a fundamental challenge in ecology, whether an organism is parasitic (Antonovics et al., 2017; Nunn & Altizer, 2006) or commensal (Kuthyar et al., 2019). Parasites often take advantage of multiple transmission pathways to infect susceptible hosts. For example, sexual contact was the predominant transmission mechanism during the 2022 mpox outbreak (Allan-Blitz et al., 2023), but cases of non-sexual skin-to-skin contact were also documented and the role of fomite and respiratory transmission were initially unclear (Beeson et al., 2023). In contrast, Zika virus is predominantly a vector-borne parasite, spread via *Aedes aegypti* and *Aedes albopictus*, but evidence emerged during the 2015–2017 outbreak that it can also be transmitted through sexual contact (Counotte et al., 2018). Commensal microorganisms also spread through multiple transmission pathways. These organisms can spread vertically from parent to offspring during childbirth and likely spread horizontally among peers in a social group (Dominguez-Bello et al., 2010; Tung et al., 2015). These varied transmission pathways can affect the course of an epidemic (Kollepara et al., 2023), the health of future generations (Rosenberg & Trevathan, 2018), and public health approaches to prevent disease transmission.

To disentangle the transmission pathways of any organism, we need a clear vocabulary for talking about the mechanisms (or modes) through which an organism moves between individual hosts. Transmission can occur directly from one host to another, indirectly through the environment, or through a vector (Nunn & Altizer, 2006). Direct transmission mechanisms include sexual and non-sexual close contact as well as airborne droplets produced during respiration, coughing, or sneezing. Indirect transmission mechanisms include shared contact with contaminated water, soil, and surfaces (fomites), and vector transmission often occurs through biting arthropods, such as mosquitos, fleas, and ticks. Each of these transmission mechanisms can facilitate the spread of organisms from non-human animals to humans (zoonosis), from humans to non-human animals (reverse zoonosis), and among conspecifics.

Determining transmission pathways is challenging, but at the heart of all these pathways are connections among individuals (or networks) comprised of social contacts, shared environments, or contact with other animals. Network analysis provides a toolkit for leveraging social and ecological data to disentangle these multiple complex infectious disease transmission dynamics (Silk et al., 2017). Networks can be used to represent different types of relations that capture specific modes of transmission. When combined with individual-level infection data, the transmission potential for a specific microorganism can be assessed (Kauffman et al., 2022; Pilosof et al., 2015; VanderWaal et al., 2016). For example, spatial data can be used to construct a network where individuals (nodes) are connected (share an edge) if they encounter each other while wearing a GPS tracking device, or if they visit the same areas (for environmental transmission networks) (Kauffman et al., 2022). Alternatively, people can name individuals with whom they work or socialize (Wasserman & Faust, 1994).

Once networks for different hypothesized transmission pathways are constructed, several statistical network methods are available to investigate which transmission pathways are most relevant for specific parasites. These network-based approaches move beyond standard methods that assume homogenous social mixing to methods that explicitly take into account social structure and heterogeneity in contact patterns (Bansal et al., 2007). One common network-based approach is to use centrality scores (i.e., a measure of the number of connections an individual has in a network) to test whether more connected individuals have a higher probability of infection (Rimbach et al., 2015; VanderWaal et al., 2014). However, this approach does not capture transmission along edges, and centrality measures are often correlated with other factors that influence infection risk, like age and social status (VanderWaal et al., 2016).

Here, we use network autocorrelation models that account for multiple network dependencies within a generalized linear modeling framework (Doreian, 1990; Leenders, 2002). This approach is useful for assessing whether the infection status of an individual's alters (their connections on a network) affect their probability of infection (Silk et al., 2017). We also use an approach called the network *k*-test, which is a permutation-based method that assesses whether infections are clustered on nodes that share edges to a greater extent than

would be expected if infections were randomly distributed on a network (VanderWaal et al., 2016). These analytical tools can be used to demonstrate the relative importance of networks that represent different transmission pathways.

Prior applications of network analysis to questions of unknown transmission dynamics have yielded positive results. For example, many gastrointestinal parasites are typically thought to be indirectly transmitted through shared environmental exposures to contaminated water or soil, but a study of *Escherichia coli* infection among brushtail possums found that a close contact network reflecting brief nocturnal encounters better predicted *E. coli* strain sharing than day-long den sharing (Blyton et al., 2014). Similarly, transmission potential networks helped uncover the role of close contact in the transmission of gastrointestinal parasites among brown spider monkeys (Rimbach et al., 2015), the transmission of *Salmonella* among Australian sleepy lizards (Bull et al., 2012), and the transmission of *E. coli* among giraffes (VanderWaal et al., 2014). In contrast, a study of people in Bangladesh found that spatial networks reflecting household proximity and shared environmental exposure better predicted cases of cholera and shigellosis than kinship networks (Emch et al., 2012).

Networks can also be used to understand how multiplexity, or the existence of multiple types of relations, influences transmission dynamics, where some ties (e.g., those representing sexual contact) might facilitate the wider spread of a parasite while others (e.g., needle sharing) might reinforce infection risk in the core of a network (Adams et al., 2013). These insights are invaluable to building an accurate understanding of infectious disease ecology and capturing the fundamental relational processes through which microorganisms spread.

In addition to contact patterns, the distribution of infectious diseases in a population is affected by broader social processes (Barrett et al., 1998; Singer & Clair, 2003). For example, cultural norms related to gender may impact how exposure differs between men and women. In a study of movement patterns among Hadza hunter-gatherers in Tanzania, men tended to travel farther for everyday subsistence tasks than women (Wood et al., 2021). Differences in daily travel distance and time spent at home likely influence both the types of parasites people encounter and the frequency with which they encounter them. In rural South Africa, a study of gender roles found that women tended to be caregivers for ill family members, suggesting that they are more likely to be exposed to directly transmitted parasites through close contact; in contrast, men tended to hunt and slaughter wildlife, suggesting that they are more likely to be exposed to zoonotic parasites (Coyle et al., 2020). Cultural context can thus be critical to understanding how characteristics measured at an individual level, like gender, can affect patterns of infectious disease at a population level.

Rapid political-economic transitions that affect peoples' livelihoods are also known to transform disease ecology (Baker et al., 1986; Goodman & Leatherman, 1998; Shephard & Rode, 1996), and research focused on social transitions has provided important insights into how political-economic forces shape health disparities (Hoke & Leatherman, 2019; Leonard et al., 2009). Market integration—the transition from subsistence to market-based livelihoods—is one type of social transition that changes disease ecology. Although most work on market integration has emphasized the effects of changing diets and activity patterns on cardiometabolic health (Lea et al., 2020; Liebert et al., 2013; Mattison et al., 2022; Swanson et al., 2023), a few studies have also shown its impact on infectious disease. For example, living closer to market centers and in houses constructed of more purchased materials (e.g., metal and concrete) have been linked with a lower prevalence of soil-transmitted helminth infections (Cepon-Robins et al., 2014; Gildner et al., 2020; Tanner & Team, 2014). Individual-level hygiene behaviors like handwashing can also play an important role in determining whether exposure leads to an active infection (Larson, 1988), and access to hygiene products like soap can be a factor that motivates individuals to participate in market economies (Godoy et al., 2005). Characterizing dynamic social processes like market integration is vital to understanding the transmission pathways that influence the spread of infectious disease.

In this study, we use methods from network analysis and anthropology to disentangle the transmission pathways associated with *Blastocystis* infection in a population undergoing rapid market integration. Prevalence of *Blastocystis* ranges from 1% to 23% in high-income countries and 22% to 100% in low and middle-income countries (Seyer et al., 2017). Despite being widespread, much remains unknown about its pathogenicity and transmission dynamics. *Blastocystis* is a genetically diverse protozoan, with at least 22 genetically distinct subtypes (STs) identified (Deng et al., 2021; Gentekaki et al., 2017). Experimental studies in rodent models show that STs differ in their pathogenicity, with some being asymptomatic and others evoking a robust inflammatory response and the formation of intestinal polyps (Ajjampur & Tan, 2016). *Blastocystis* is also a generalist, colonizing a wide range of non-human hosts, including pigs, cattle, dogs, chickens, and non-human primates (Hublin et al., 2021). Its genetic diversity, varying pathogenicity, and ability to colonize a diverse range of hosts make it difficult to identify clear pathways that lead to human infection.

Blastocystis is transmitted fecal-orally, but the pathways that lead to fecal-oral transmission among humans have been difficult to document. Prior studies have suggested that *Blastocystis* may be transmitted through close contact, but it is difficult to differentiate exposure that occurs through the contamination of shared living spaces versus direct contact (Pipatsatitpong et al., 2012; Yoshikawa et al., 2000). Several studies ranging geographically from Southeast Asia to Africa to Europe found evidence of waterborne transmission via contaminated drinking water (Angelici et al., 2018; Anuar et al., 2013; Poulsen et al., 2016). It has also been suggested that *Blastocystis* can be transmitted zoonotically, although there is conflicting evidence for this transmission pathway, which might differ by subtype (ST) (Greige et al., 2019; Jinatham et al., 2021; Paulos et al., 2018; Scanlan et al., 2016).

To investigate different *Blastocystis* transmission pathways in low-resource settings, we used three networks representing human-to-human close contact transmission, environmental transmission, and zoonotic close contact transmission in rural northeast

Madagascar. We tested three hypotheses related to these transmission potential networks. First, we hypothesized that friendships that entail spending free time together would facilitate human-to-human transmission through close contact. Second, we hypothesized that close household proximity would result in exposure to shared environments and thus facilitate environmental transmission. Third, we hypothesized that ownership of domesticated animals would facilitate zoonotic transmission.

We also investigated whether individual characteristics of our study participants affected their risk of infection with *Blastocystis*, which if so, would influence the predictive capacity of the networks for infection patterns. We hypothesized that market-based livelihoods—indexed by ownership of durable commercial goods, household infrastructure, and cash crop (vanilla) production—and washing hands with soap would be associated with a lower probability of infection, and thus would moderate the effect of each of the three contact networks. In these models, we also considered the effect of age, gender, and village, all of which may influence exposure and susceptibility to infection (Nunn & Altizer, 2006).

2 | MATERIALS AND METHODS

2.1 | Study setting

Study participants were recruited from three villages on the border of Marojejy National Park in the SAVA region of northeast Madagascar. People living in this region are primarily agriculturalists, producing a mix of rice as a subsistence crop and vanilla as a cash crop, along with fruits and vegetables that are grown for home consumption and sale at markets. Reliance on vanilla production increased substantially in the 1980s and 1990s when Madagascar passed market liberalization policies that encouraged commercial agriculture (Laney & Turner, 2015). However, subsistence agriculture persists throughout the region (Laney & Turner, 2015), and this diversity in agricultural practices contributes to a mixed economy where there is wide variation in the degree to which individuals' livelihoods are integrated with the market economy. Even among farmers who produce vanilla, crop yields are highly variable (Celio et al., 2023; Hänke et al., 2018). This variability is associated with household-level differences in cash availability and food security (Herrera et al., 2021; Laney & Turner, 2015), which may influence susceptibility to infectious diseases.

In low and middle-income countries, rural farmers' close interaction with both land and animals puts them at a high risk of infectious disease (Rohr et al., 2019). The risk of zoonotic infectious disease in this region of Madagascar is mostly due to substantial interaction with domesticated animals (dogs, pigs, cows) and rodents (Nunn et al., 2022). The people have substantially less and minimal contact with wildlife, including non-human primates. At the time of data collection, two of the three study villages had wells. All three villages are located near streams and rivers that are regularly used for gathering drinking water, bathing, and washing clothes and may be a source of environmentally transmitted parasites.

2.2 | Data collection

The data used in this analysis were collected between 2019 and 2023. Surveys were administered to 1297 adult members of farming households across the three study villages in the local Malagasy dialect by a trained research team over three field seasons that encompassed both dry and wet season months. The survey was administered with Samsung tablets using Qualtrics software (Qualtrics, Provo, Utah). Participants were compensated with mobile phone credits. A subset of participants ($n = 804$) provided fecal samples for DNA metabarcoding analysis. We also collected information on ownership of domesticated animals, and fecal samples were obtained from 133 domesticated animals owned by the 804 participants with fecal samples. Dogs, cats, pigs, and cows were included in the study. We screened rat, mice, and shrew feces that were found in and around participants' houses. All human study participants were 18 years or older at the time of data collection and provided informed consent. Study protocols were approved by the Duke University Institutional Review Board (Duke IRB Number 2019–0560), the Malagasy Ethics Panel (Permit Number 114 MSANP/SG/AGMED/CERBM), the Malagasy Ministry of Environment (No. 280/19, 57/20, 191/20, 307/21, 048/22, 33/22, 321/23 MEEF/SG/DGF/DSAP/SCB), and the Institutional Animal Care and Use Committee (Protocol Registry Number A178-22-10).

2.3 | Social networks and socioeconomic characteristics

To construct the social networks used in this analysis, we asked each participant to name up to five people with whom they spend their free time (hereafter referred to as "friendship"). Participants shared an edge on the friendship network if they named or were named by another participant who was surveyed (i.e., if at least one member of the pair nominated the other). We used snowball sampling to recruit study participants, starting with an initial set of individuals in each village who owned agricultural land and then enrolling people they named (Naderifar et al., 2017). During each survey, we also recorded the GPS coordinates of participants' homes to construct a weighted network representing house proximity based on Euclidean distance. To construct the human-domesticated animal interaction network, each participant identified individual domesticated animals that they owned, and we constructed a bipartite network with each individual human connected to individual domesticated animals. Each participant also reported their age, gender, highest level of education, and main occupational activity. Additionally, we asked participants what they mainly use to wash their hands, with response options of soap and water or water only.

We used three measures to capture participants' degree of market integration, including production for the market and consumption from the market (Lu, 2007). We asked whether participants grew vanilla in the past year (yes or no) because vanilla is the major source of cash income in the region and rarely used for cooking at home

(Laney & Turner, 2015). We also used two measures to assess the degree to which participants' households purchased market goods. A commercial goods index was created to measure the number of durable goods a household owned, capturing ownership of cell phones, televisions, bicycles, motorcycles, generators, refrigerators, and computers (Bindon et al., 1997; Henrich, 1997). Values for the commercial goods index range from 0 (no durable goods owned) to 7 (all durable goods owned).

We used a household material lifestyle index to measure the degree to which an individual's house was constructed of purchased materials (Liebert et al., 2013). To construct the index, we created a scale that ranked each component of house construction, taking into account both the ability to gather the material locally and the intensity of resources needed to purchase the material (e.g., an individual could buy and transport metal sheets themselves, but cement requires a more intensive construction process in this setting). For floors, construction with dirt or locally acquired plants (bamboo, rafia, or ravinala) received a value of zero; construction with wood planks received a value of one; and construction with cement received a value of two. For walls, construction with locally acquired plants, mud, or compacted earth received a value of zero; construction with wood planks received a value of one; construction with bricks received a value of two; construction with metal sheets received a value of three; and construction with cement received a value of four. For roofs, construction with locally acquired plants received a value of zero, construction with metal sheets received a value of one, and construction with cement received a value of two. We then summed the standardized values for each house component to construct the household lifestyle index, with higher values indicating houses constructed of more purchased materials.

2.4 | *Blastocystis* infection status

We used DNA metabarcoding to screen for *Blastocystis* infection. Fecal samples were preserved in nucleic acid preservation (NAP) buffer (Camacho-Sanchez et al., 2013) or Zymo DNA/RNA Shield (Zymo Research, Irvine, California) and transported from the field site to the University of California Santa Barbara where analyses took place. Samples were kept at room temperature at the field site, stored at −20°C in Madagascar, and then brought to room temperature for transportation to Santa Barbara where they were stored again at −20°C until DNA extraction. Two different storage solutions (NAP buffer and Zymo DNA/RNA Shield) were used due to complications with lab supplies during the COVID-19 pandemic. Comparing *Blastocystis* prevalence between the two solutions revealed no statistically significant differences ($p = 0.12$).

We first extracted DNA from fecal samples using Zymo MiniPrep Fecal kits (Zymo Research, Irvine, California), and PCR was used to amplify the DNA with a G4 primer set for 18S ribosomal DNA (Gogarten et al., 2020; Krogsgaard et al., 2018). Forward and reverse primers contained 8-nucleotide barcodes with a Hamming distance of at least 4. PCR reactions were carried out in 15 uL volumes, each consisting of: 3 uL of each forward and reverse primer (2 uM stock concentration); 7 uL from a Mastermix comprised of 0.7 uL of Amplitaq Gold polymerase, 150 uL MgCl2, 150 uL Amplitaq Gold buffer, 12 uL BSA, 6 uL DMSO, and 344 uL water; and up to 2 uL template DNA (1–100 ng total). Cycling conditions were 10-minute hot-start activation, 35x cycles of 15 s at 95°C, 30 s at 57°C, 40 s at 72°C, and a final 5-min extension at 72°C. DNA concentrations were then measured, pooled, normalized, and purified using MinElute columns prior to multiplexing with additional libraries. The final library was sequenced three times on an Illumina MiSeq (v3 2 × 300 bp, 25 M reads) at the UC Davis Genome Center. Sequences were demultiplexed using cutadapt with zero error tolerance (Martin, 2011). We used the dada2 bioinformatics pipeline (Callahan et al., 2016) to filter and trim amplicons (minimum length = 100, 15% PhiX removed), remove errors, dereplicate, infer amplicon sequence variants (ASVs) using the pseudo-pooling method, merge pairs and concatenate in cases when there was no overlap (Gogarten et al., 2020), and remove chimeras.

The relative read abundance of each ASV was calculated. ASVs that accounted for less than 2% of a sample's relative read abundance were removed to avoid potential sequencing errors or tag jumps. We assigned taxonomy to all ASVs using both the assignTaxonomy function in dada2 and the IdTaxa function in the *DECIPHER* package in R (Murali et al., 2018). All candidate *Blastocystis* ASVs were then queried in the NCBI GenBank database to retrieve STs using the blastn function. We calculated the mean percent identity for each taxonomic identification from the top 100 hits. The ST with the highest percent identity was then assigned to each ASV. *Blastocystis* variants that could not be matched to specific STs were categorized as "unknown STs" in the analysis.

2.5 | Statistical analysis

All analyses were conducted in R version 4.3.1 (R Core Team, 2023). We computed descriptive statistics to characterize demographics, handwashing behavior, market integration, and *Blastocystis* prevalence across the three study villages. The friendship, house proximity, and human-domesticated animal networks were constructed and characterized using the *igraph* package (Csárdi et al., 2023). The friendship network and human-domesticated animal network were unweighted, and edges in the house proximity network were weighted by the inverse geographic distance between houses, with larger edge weights indicating more proximate households. To investigate the relationship between the friendship network and the house proximity network—that is, to assess whether participants tended to name their neighbors as someone they spend their free time with—we conducted multiple regression quadratic assignment procedures (MRQAP) with 1000 iterations for each village using the *sna* package (Butts, 2023; Martin, 1999). We created sociograms using the *ggraph* package (Pedersen & RStudio, 2022).

We used two analytic approaches to assess the relationship between the friendship and house proximity networks and *Blastocystis*

infection. First, we used network autocorrelation models, which incorporate a term that accounts for network-based dependencies of the response variable (infection status) in a generalized linear modeling framework (Doreian, 1990; Leenders, 2002). For the friendship network, the autocorrelation term captures the proportion of friends who are infected with *Blastocystis*. For the house proximity network, the autocorrelation term captures the proportion of weighted connections who are infected (i.e., the summed weights of infected connections divided by the summed weights of all connections). Each model included relevant demographic predictors that could influence infection risk (age, gender, and village), handwashing behavior, and market integration measures that capture both wealth and hygiene-related dimensions of rural livelihoods that could influence infection risk (vanilla production, commercial goods index, and household lifestyle index). To test our hypothesis that handwashing behavior and market integration would moderate the network effects on patterns of infection, we included interaction terms for handwashing and each network autocorrelation term as well as the market integration indices and each network autocorrelation term. Continuous variables were standardized prior to analysis. We compared the predictive performance of models with different sets of these variables using sample-size adjusted Akaike Information Criterion (AICc) (Burnham & Anderson, 2002; Symonds & Moussalli, 2010). Weighted coefficients were then computed across a set of models that had a delta AICc less than two (hereafter called component models). Model comparison and averaging were conducted using the *MuMIn* package (Bartoń, 2023).

For the second approach, we used the network *k*-test, which is a permutation test in which infection status is randomly assigned using the same underlying network structure (VanderWaal et al., 2016). We considered the observed networks to reflect *Blastocystis* transmission potential if the mean number of infected nodes within one step of an infected node (*k*-statistic) exceeded the *k*-statistic in 95% of the permuted networks. For the spatial house proximity network, we conducted permutation tests with a range of distance thresholds for setting the existence of an edge (between 5 and 100 meters at 5-meter intervals), which varies the number of nodes that are considered within one step of the focal node. Each *k*-test was performed using 1000 permutations.

3 | RESULTS

3.1 | Participant characteristics

Of the 1297 participants across three villages, 804 (62%) provided fecal samples and were included in the analysis. These 804 participants had a median (interquartile range [IQR]) age of 35 (25–49) years, and 48% ($n = 385$) were women. Most participants reported at least some education, with only 4% ($n = 32$) reporting no education. Nearly all (91%, $n = 728$) participants reported farming as their primary occupation. Approximately half (54%, $n = 436$) of participants reported that they wash their hands with soap versus washing their hands with water only.

The median (IQR) household market integration index value was −0.31 (−1.51–1.85), indicating that most people tended to construct some but not all of their house with purchased materials. Overall, participants owned a median (IQR) of 1 (1–2) commercial goods, suggesting few people purchase durable commercial goods. Vanilla production was common overall, with 82% ($n = 656$) of participants reporting that they grew at least some vanilla in the past year (Table 1).

3.2 | Social network characteristics

Village C (estimated village population size = 1900) had the largest friendship network, with 393 nodes and 689 edges, while the networks for Village A (estimated village population size = 2700) and Village B (estimated village population size = 900) included 191 nodes (270 edges) and 220 nodes (228 edges), respectively (Figure 1). MRQAP results indicated that the spatial and social networks were not statistically significantly associated for Village A ($R^2 < 0.01$, $p = 0.924$), Village B ($R^2 < 0.01$, $p = 0.943$), or Village C ($R^2 < 0.01$, $p = 0.945$). Friendship network density was similar across the three villages, with Village A being the most dense (0.7% of all possible ties existed), followed by Village B (0.5% of all possible ties) and Village C (0.4% of all possible ties). Village C had the highest mean degree centrality (mean [SD]: 3.5 [2.2]), followed by Village A (mean [SD]: 2.8 [2.0]) and Village B (mean [SD]: 2.1 [1.7]). Transitivity followed a similar pattern, with Village C having the highest probability of a triad being closed (0.2), followed by Village B (0.14) and Village A (0.14).

3.3 | Patterns of *Blastocystis* infection

Among all participants, 74% ($n = 598$) were positive for at least one ST of *Blastocystis*. Village C and B had the same prevalence at 78% (Village C: $n = 305$, Village B: $n = 171$). Village A had a lower prevalence (64%, $n = 122$). ST3 was most prevalent across all three villages (39%, $n = 317$), followed by ST1 (35%, $n = 281$), the unknown STs (24%, $n = 195$), and ST2 (18%, $n = 147$) (Figure 2).

To assess demographic and network predictors of *Blastocystis* infection, we modeled five separate outcomes: probability of infection with any *Blastocystis* ST and probability of infection with each of the four *Blastocystis* STs. Model averaged coefficients for the first set of models predicting any infection suggested that close proximity to infected households was the most important predictor of infection (Figure 3). That is, a one standard deviation increase in the proportion of connections to infected households was associated with 56% higher odds of infection with any *Blastocystis* ST (model averaged OR [95% CI]: 1.56 [1.33–1.82]). The network autocorrelation term for household proximity was included in each of the 13 component models with a delta AICc less than two in this set (summed AICc weight = 1). In contrast, the association between proportion of infected friends and infection with any *Blastocystis* ST was close to zero (model averaged OR [95% CI]: 0.95 [0.81–1.10]), and the

TABLE 1 Participant characteristics by village.

Characteristic	Village			
	Village A $N = 191$	Village B $N = 220$	Village C $N = 393$	All villages $N = 804$
Age, Median (IQR)	41 (31–54)	30 (23–47)	34 (25–48)	35 (25–49)
Gender, *n* (%)				
Women	95 (50%)	96 (44%)	194 (49%)	385 (48%)
Men	96 (50%)	124 (56%)	199 (51%)	419 (52%)
Education, *n* (%)				
None	11 (5.8%)	11 (5%)	10 (2.5%)	32 (4%)
Primary	115 (60%)	84 (38%)	170 (43%)	369 (46%)
Secondary	51 (27%)	102 (46%)	157 (40%)	310 (39%)
Higher	14 (7.3%)	22 (10%)	50 (13%)	86 (11%)
Unknown	0 (0%)	1 (0.5%)	6 (1.5%)	7 (0.8%)
Primary occupation[a], *n* (%)				
Farmer	187 (98%)	197 (90%)	344 (88%)	728 (91%)
Non-Farmer	4 (2%)	23 (10%)	49 (12%)	76 (9%)
Wash hands with soap (vs. water only), *n* (%)	58 (30%)	148 (67%)	230 (59%)	436 (54%)
Household lifestyle index, median (IQR)	−1.51 (−1.51–1.85)	0.65 (−0.55–1.85)	0.65 (−1.55–1.85)	−0.31 (−1.51–1.85)
Commercial goods owned, median (IQR)	1 (1–2)	1 (1–2)	1 (1–2)	1 (1–2)
Vanilla grown, *n* (%)	145 (76%)	182 (83%)	329 (84%)	656 (82%)

[a]Non-farming primary occupations included carpenter, driver, laborer, shopkeeper, student, teacher, unemployed, and other/unknown.

network autocorrelation term for the friendship network was only included in six of the 13 the component models (summed AICc weight = 0.43) (Table S1).

In analyses of the different STs, close proximity to infected households was only informative in analyses of ST3 (model averaged OR [95% CI]: 1.02 [1.00–1.03]) (Table S2). In the model for ST3, age and gender were also informative predictors, with a one standard deviation increase in age being associated with 26% higher odds of ST3 infection (model averaged OR [95% CI]: 1.26 [1.08–1.45]), and men having 31% lower odds of ST3 infection than women (model averaged OR [95% CI]: 0.69 [0.52–0.92]). In analyses of ST2, age was the only informative predictor, but in the opposite direction, with a one standard deviation increase in age being associated with 24% lower odds of ST2 infection (model averaged OR [95% CI]: 0.76 [0.62–0.92]). None of the variables we included in our modeling were informative predictors of ST1 infection, and the best performing model (determined by AICc) only included handwashing behavior (i.e., washing hands with soap versus water only) and village. The most informative predictor of infection with the unknown STs was village, with participants in Village B and Village C having higher odds of infection than participants in Village A (model averaged OR [95% CI]: 2.31 [1.33–4.02] and 3.16 [1.91–5.20], respectively). Interaction terms for the market integration indices, handwashing behavior, and network autocorrelation terms were not informative in any models and thus excluded from the final model set.

To further assess the spatial patterning of *Blastocystis* infection and to gain more precise insight into the scale at, which house proximity matters for infection, we performed network *k*-tests. At distance thresholds between 5 and 25 meters, results of the *k*-tests for household proximity in each village and for each *Blastocystis* ST were statistically significant (Figure 4). For all STs, the *k*-tests were statistically significant up to a distance threshold of 60 meters for Village B and 100 meters for Village A. The friendship network *k*-tests were not statistically significant for any village and *Blastocystis* ST combination.

3.4 | Subgroup analysis to identify zoonotic transmission potential

We determined *Blastocystis* infection patterns among humans and the domesticated animals they owned to assess zoonotic transmission potential (Figure 5). Among the 133 domesticated animals owned by the 804 human participants, only 27 (20%) were infected with *Blastocystis*. Most of the infected domesticated animals were pigs infected with ST5 ($n = 25$), which was not found in any of the people we sampled. One dog was infected with ST3, but their owner was not infected with ST3. One cow was infected with ST10, but no humans were infected with ST10. Given that no human-domesticated animal dyads were infected with the same STs, we did not perform additional statistical analyses to assess whether interaction with domesticated animals predicted human infection. Rat feces collected in and around participants' houses were positive for ST4 but not any of the STs identified in human samples.

4 | DISCUSSION

Although *Blastocystis* spp. is one of the most common human intestinal protozoans, its transmission dynamics remain unclear

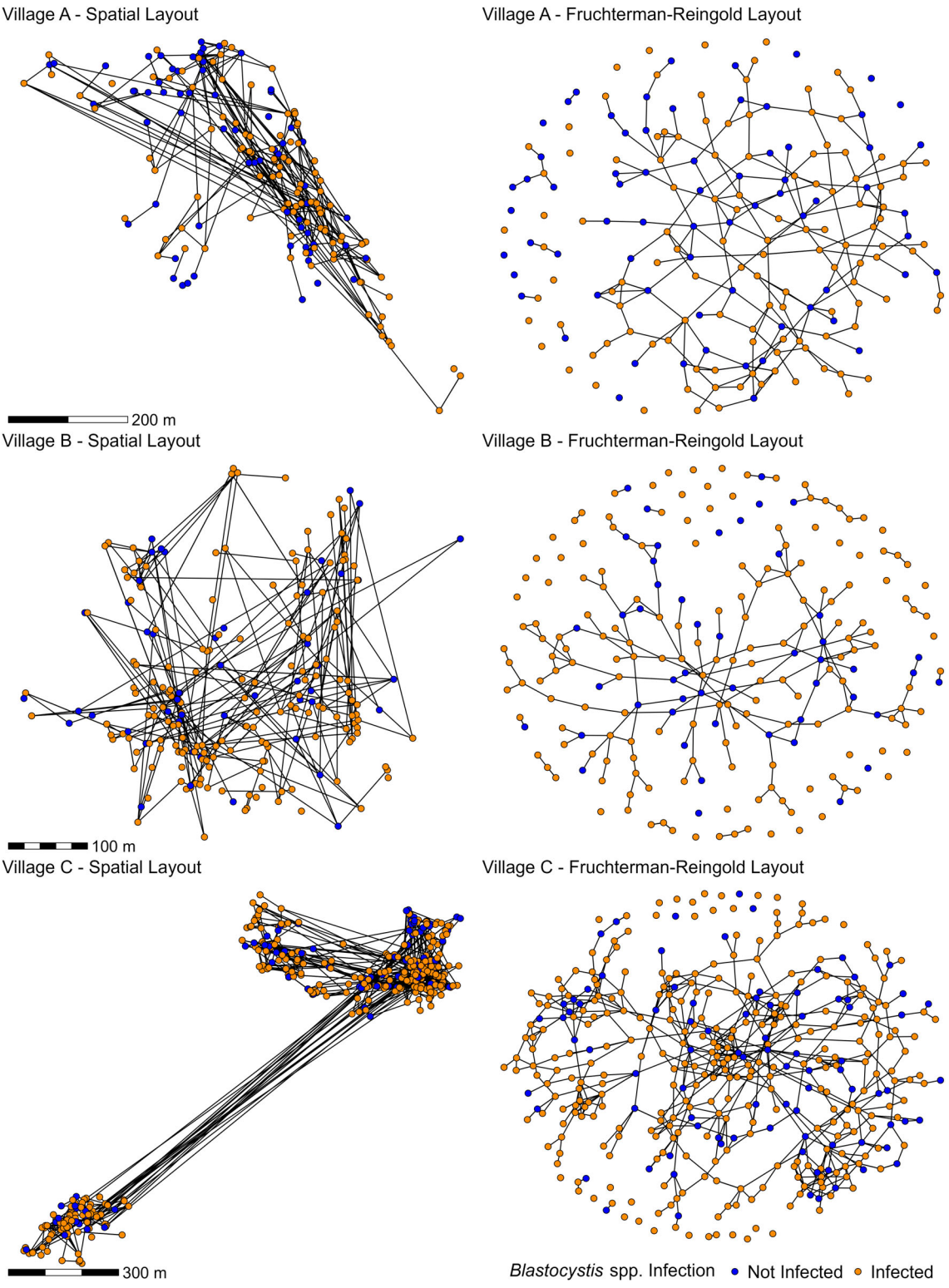

FIGURE 1 Friendship networks for Village A ($n = 191$), Village B ($n = 220$), and Village C ($N = 393$). Nodes represent individual participants, and two participants share an edge if at least one individual in the dyad named the other individual. Networks in the left column are plotted based on the GPS coordinates of participants' houses, and networks in the right column are plotted based on a spring-embedded Fruchterman–Reingold algorithm. Node color indicates *Blastocystis* infection status (blue: No infection; orange: Infection with any of the four subtypes). Associations between the house proximity and friendship networks were assessed using multiple regression quadratic assignment procedure and were not statistically significant for Village A ($R^2 < 0.01$, $p = 0.924$), Village B ($R^2 < 0.01$, $p = 0.943$), or Village C ($R^2 < 0.01$, $p = 0.945$), indicating that participants did not tend to name their neighbors as friends in each of the three villages.

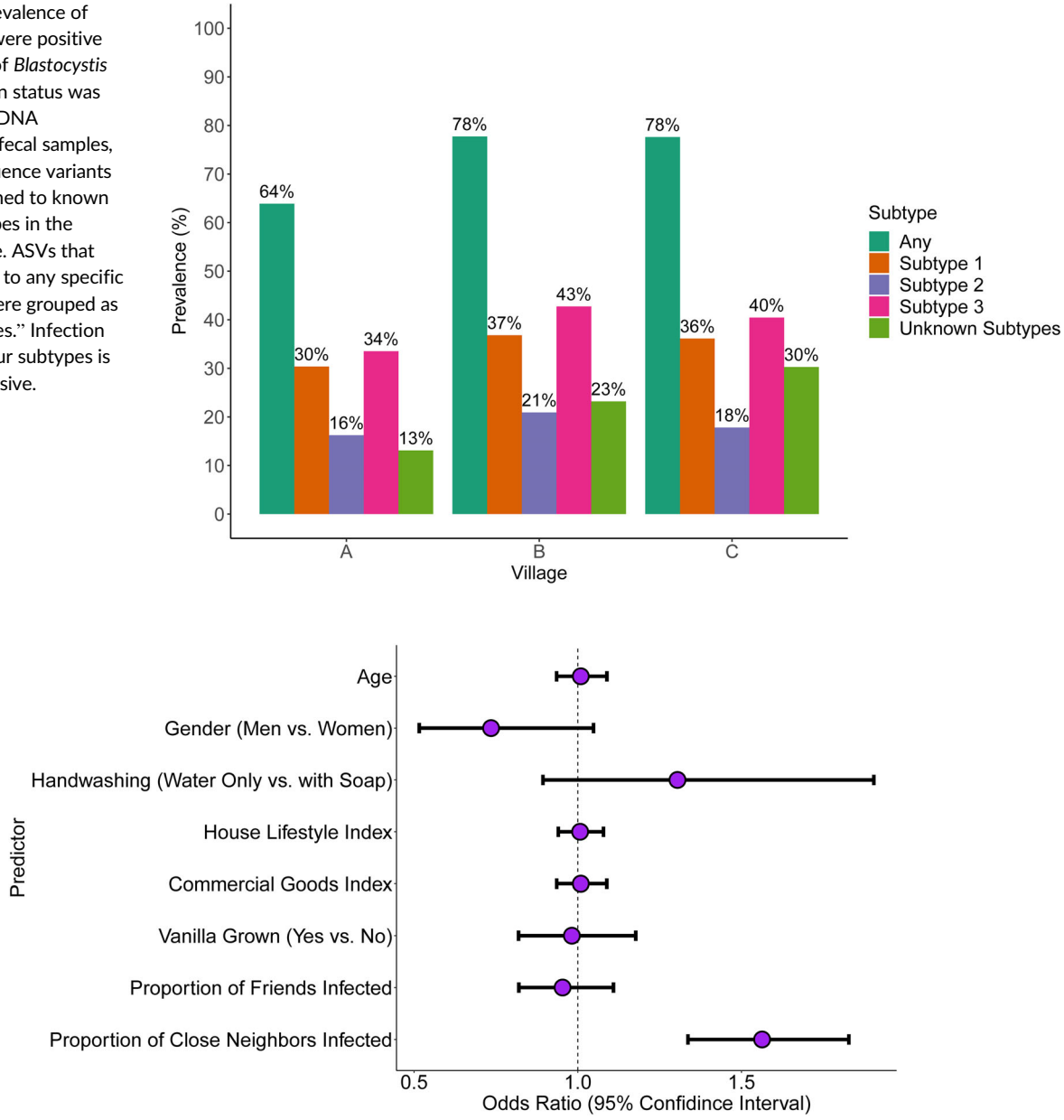

FIGURE 2 Prevalence of participants who were positive for each subtype of *Blastocystis* by village. Infection status was determined using DNA metabarcoding of fecal samples, and amplicon sequence variants (ASVs) were matched to known *Blastocystis* subtypes in the GenBank database. ASVs that were not matched to any specific known subtype were grouped as "unknown subtypes." Infection with any of the four subtypes is not mutually exclusive.

FIGURE 3 Association between predictor variables and odds of infection with any *Blastocystis* subtype in rural northeast Madagascar. Individuals with more infected proximate neighbors had higher odds of infection. Age, gender, handwashing behavior, market integration, and proportion of infected friends were not significantly associated with infection. Points and error bars represent odds ratios and 95% confidence intervals computed from model averaging of candidate logistic regression models with a delta AICc less than two (Table S1).

(Jinatham et al., 2021). In this study, we used transmission potential networks to disentangle the roles of social, environmental, and zoonotic transmission pathways for the spread of *Blastocystis* in rural northeast Madagascar. We found that proximity to infected households predicted infection—particularly for ST3—and that village predicted infection for ST2 and unknown STs. In contrast, spending free time with infected individuals did not predict infection with any ST, and no humans were infected with the same ST as the domesticated animals they owned. We did not find evidence that market integration was associated with *Blastocystis* infection. Overall, these findings suggest that environmental transmission is the most important transmission pathway for *Blastocystis* in this setting and that *Blastocystis* is more host specific than previously thought. Below, we review the implications of our findings for each transmission pathway alongside evidence from other contexts.

Most evidence for human-to-human transmission comes from relatively controlled settings, such as healthcare or childcare facilities (Pipatsatitpong et al., 2012; Yoshikawa et al., 2000), and other studies have found no evidence of human-to-human close contact transmission. For example, a study of *Blastocystis* infection among US households found that each infected individual in the study was in a different household, meaning no two household members were both

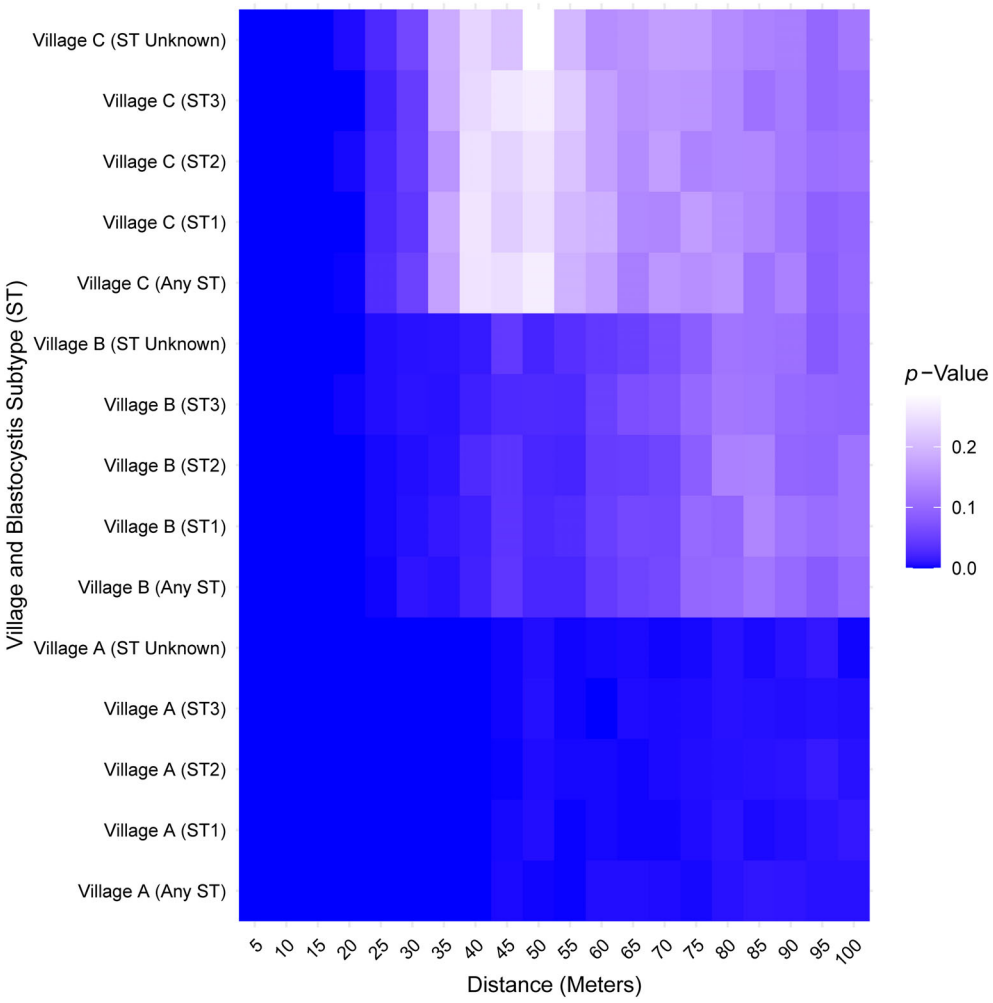

FIGURE 4 *Blastocystis* transmission potential of household proximity networks in three villages in northeast Madagascar. Each row shows the transmission potential of the household proximity networks for each village and *Blastocystis* ST combination across distance thresholds ranging from 5 to 100 m. The color of each tile shows the results of the network *k*-test for the given village, ST, and distance threshold. Darker blue tiles represent lower empirical *p*-values, and lighter tiles represent higher *p*-values. Because the generally observed trends are towards higher *p*-values at larger distance thresholds, we have not undertaken any correction for multiple comparisons.

infected (Scanlan et al., 2016); importantly, this study was conducted in a high-income country with modern sanitation and only found a 7% prevalence among study participants. In contrast, in a study of *Blastocystis* among rural Indigenous communities in Malaysia, having an infected family member was one of the strongest predictors of infection, but drinking untreated water also predicted infection (Anuar et al., 2013). In these cases, it is difficult to disentangle the effect of shared environmental exposure (e.g., to untreated drinking water) and direct human-to-human transmission. In our study, we did not find evidence that spending free time with an infected individual was associated with infection. This finding leads us to believe that the importance of house proximity networks for predicting infection is related to shared environmental exposures rather than socializing with neighbors.

Our findings provide evidence for environmental transmission of *Blastocystis* at two scales, both within and among villages. Within each village, we found that close proximity to an infected household predicts infection. The network *k*-tests indicated that the proximity at which this pattern holds differs across villages, ranging from 25 meters in Village C to 100 meters in Village A. These differences may be related to shared water sources and how water acquisition clusters within each village (e.g., differences in available water sources across villages and how households cluster around different water sources). Prior studies have found strong evidence in support of contaminated water as a source of *Blastocystis* infection. A case study of a patient suffering gastrointestinal distress in Italy provides the most direct evidence, where clinical laboratory results showed that *Blastocystis* sampled from the patient and a non-potable water source from which he drank were both ST1 (Angelici et al., 2018). Live *Blastocystis* cysts have also been documented in wastewater treatment facilities in Malaysia, Scotland, and the Philippines (Banaticla & Rivera, 2011; Suresh et al., 2005). These studies highlight the importance of sanitation infrastructure for ensuring access to clean drinking water. In rural Madagascar, access to safe drinking water is severely limited and use of water infrastructure is affected by travel time between peoples' homes and water sources (Boone et al., 2011). The tendency to use closer water sources further suggests that proximate households likely share common water resources, but more proximate households could also share similar practices of water treatment (e.g., whether they boil water before drinking) and share latrines. Along with the importance of close proximity, village identity predicted infection with unknown STs. This finding could indicate that water access at the village level impacts patterns of *Blastocystis* infection; for example, Villages B and C had higher odds of infection with unknown STs and had wells

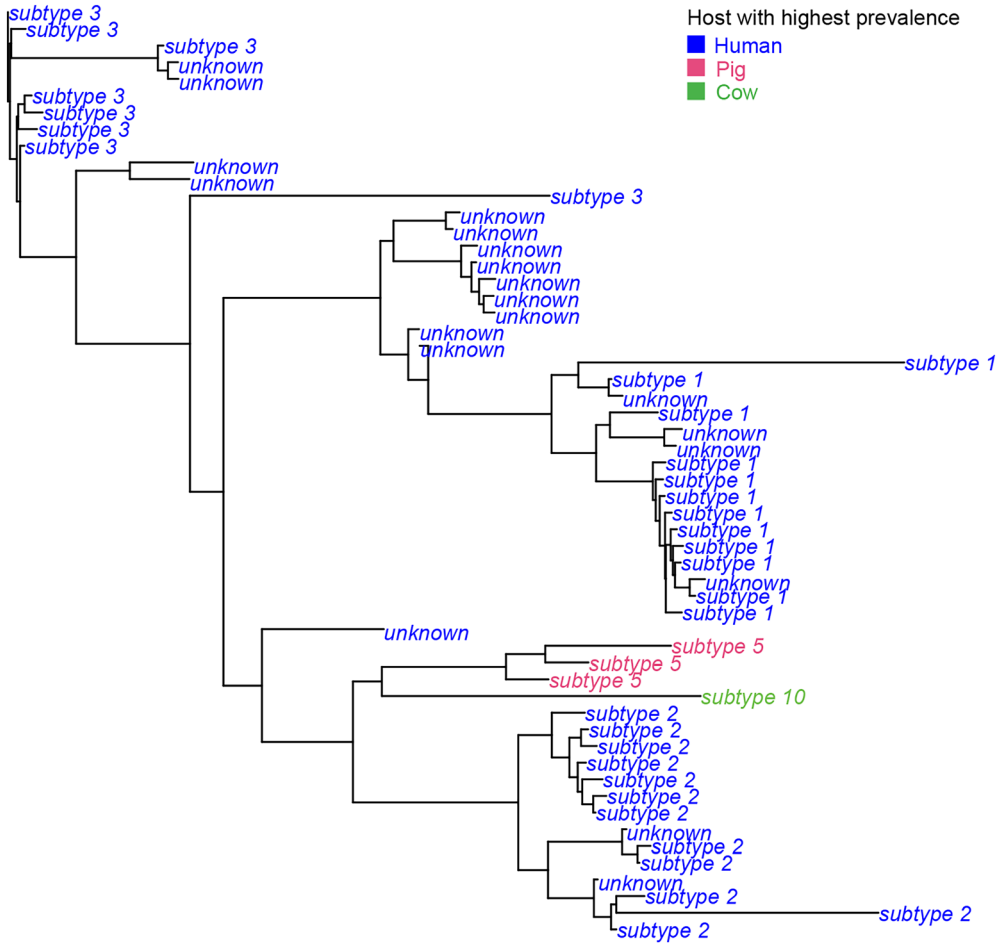

FIGURE 5 Neighbor-joining tree of *Blastocystis* amplicon sequence variants (ASVs) using the Kimura 80 + Gamma model. ASVs are classified to *Blastocystis* subtype and color-coded based on the host species with the highest prevalence for that subtype. For visibility, we grouped highly similar ASVs ($h < 0.02$) into single leaves. Leaves labeled "unknown" correspond to *Blastocystis* ASVs that did not match one particular subtype in GenBank with high similarity. Generally, subtypes 1, 2, and 3 were found in humans (blue), while subtype 5 was found in pigs (pink), and subtype 10 was found in cows (green). Subtype 3 was also found in one dog.

(in contrast to Village A). Use of well water may thus be a source of infection with unknown STs of *Blastocystis*.

Settlement patterns of the villages also differ and may play a role in explaining variation in the effect of household distance. For example, in Village C, *Blastocystis* infection is clustered at a much closer household proximity (up to 25 meters), which may be related to the village's more dispersed settlement pattern. A more dispersed settlement pattern could reduce the intensity with which spaces are used throughout a village, reducing buildup of *Blastocystis* in the environment and thus reducing risk of infection except for when households are in closer proximity. In contrast, infection status is clustered at a much more distant household proximity in Village A (up to 100 meters), possibly reflecting its more centralized settlement pattern. A more centralized settlement pattern suggests that the intensity with which space is used in the village is more uniformly distributed and thus risk of infection may also be more uniformly distributed.

We failed to find strong evidence for zoonotic transmission of *Blastocystis* in the villages included in our study. Although *Blastocystis* has been documented in 135 mammal species (not including humans), there is limited evidence that it is transmitted to humans zoonotically or transmitted to non-human animals via reverse zoonosis (Hublin et al., 2021). Each of the STs found in our human samples have been documented in domesticated animals in previous studies. For example, ST1, ST2, and ST3 have each been found in cattle living in a variety of geographic settings, including China, Lebanon, and the United States (Hublin et al., 2021). In our study, however, we did not document any of these STs in the cats, pigs, or cows sampled, and only one dog was infected with ST3.

In addition to investigating the three transmission potential networks, we identified two demographic predictors of *Blastocystis* infection: age and gender. Our results suggest that older participants and women were at a greater risk of infection with ST3, but younger participants were at a greater risk of infection with ST2. Given the likely role of environmental transmission, older adults and women may have more opportunities for ST3 exposure due to greater time spent near their houses and in the villages, whereas younger men (over the age of 18) tend to spend more time working in the agricultural fields or forests away from home, even spending nights in their fields to guard vanilla crops. During their time away from home, men likely gather their drinking water from a stream, rather than transporting it from a source in the village. If water is a primary mode of transmission for *Blastocystis*, this behavior would lead to gendered differences in exposure risk because men would have fewer opportunities for exposure to potentially contaminated water in the village. These gender norms and how they manifest in everyday activities can shape different patterns of exposure to infectious diseases and are an important consideration when designing interventions (Buckee et al., 2021;

Coyle et al., 2020). Further research is needed to unpack the potential effects of interactions among gender, age, and movement patterns on infectious disease transmission in rural Madagascar.

We did not find evidence for a relationship between market integration and *Blastocystis* infection; however, market integration can result in rapid changes that can have far reaching effects on the biocultural processes that our findings suggest are linked with *Blastocystis* infection. For example, rural agricultural communities tend to have kin-dense social networks, but social networks become less kin dense as communities become more market integrated (Colleran, 2020). These changes in network structure may influence who is most exposed to parasites that cluster among neighboring households.

Our study had several limitations worth noting. First, the cross-sectional study design limits our ability to make causal inferences about infection status. Although the exact duration of *Blastocystis* infection is unknown, evidence suggests that untreated infections could last anywhere from a few months to years (Stensvold et al., 2009). Therefore, it is unclear when participants became infected and whether there have been changes in the socioecological factors and network connections that we measured since initial infection.

Second, our study only included adults (≥ 18 years old), yet children may be important to understanding the disease ecology of *Blastocystis* in this setting, including in relation to domesticated animal contact patterns. In other low-resource settings, *Blastocystis* prevalence is particularly high among children. One study among Senegalese children in rural agricultural villages documented 100% prevalence in their sample of 93 children between 6 and 10 years old (El Safadi et al., 2014). Given that women have a more intense level of interaction with children in the study villages relative to men, a high prevalence of *Blastocystis* among children could also help explain the relatively high prevalence we found among women. Future research could elucidate the role of children in the disease ecology of *Blastocystis* in rural Madagascar and the implications for patterns of infection among adults.

Third, given the difficulty of reliably collecting fecal samples from dogs, we had a smaller number of samples relative to the total population size of dogs in the villages. However, it is not customary for dogs to live inside houses in this region, which likely reduces opportunities for transmission. Cats were also under sampled relative to the true population size of cats in villages, but they are much less common in the villages than dogs.

In conclusion, we applied cutting-edge and rigorous network science approaches to investigate *a priori* transmission mode hypotheses in a low-resource setting. With these methods, we found that *Blastocystis* infection is likely driven by environmental exposures near households and may be more host specific than previously thought. We did not collect water samples to test for the presence of *Blastocystis* in drinking water, yet our findings point to more fine-grained hypotheses that could be investigated in future research using similar methods. Overall, this study highlights the potential for generating multiple transmission potential networks to assess relevant pathways for infection where existing evidence is limited and the power of network science approaches to assess these pathways and the role of behavioral, demographic, and cultural factors. Thus, these approaches have the potential to contribute to public health action while advancing broader understanding of the socioecological drivers of infection.

AUTHOR CONTRIBUTIONS

Tyler M. Barrett: Conceptualization (lead); formal analysis (lead); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead). **Georgia C. Titcomb:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); visualization (equal). **Mark M. Janko:** Formal analysis (equal); investigation (equal); methodology (equal). **Michelle Pender:** Data curation (equal); project administration (equal). **Kayla Kauffman:** Data curation (equal); investigation (equal); methodology (equal). **Alma Solis:** Investigation (equal). **Maheriniaina Toky Randriamoria:** Project administration (equal). **Hillary S. Young:** Resources (equal); supervision (equal). **Peter J. Mucha:** Formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); supervision (equal). **James Moody:** Formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); supervision (equal). **Randall A. Kramer:** Funding acquisition (equal); investigation (equal); methodology (equal); supervision (equal). **Voahangy Soarimalala:** Funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal). **Charles L. Nunn:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (equal); supervision (lead).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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