Molecular Epidemiology of *Cryptosporidium* spp. in an Agricultural Area of Northern Vietnam: A Community Survey

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Research

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Abstract

Background

Cryptosporidium spp. is a protozoan parasite with worldwide distribution that causes cryptosporidiosis in humans and other animals. In the countryside of northern Vietnam, where free-roaming livestock are widespread, cryptosporidiosis is an important zoonotic disease. However, there have been few studies of cryptosporidiosis in Southeast Asia from the perspective of zoonotic disease epidemiology. The purpose of this study was to investigate the occurrence of *Cryptosporidium* infection in both humans and animals and to gain an awareness of the potential threat posed by this zoonotic infection in northern Vietnam.

Methods

We conducted a community survey to collect information about cases of diarrhea in an agricultural area of northern Vietnam. For this study, a total of 2715 samples (2120 human diarrheal samples, 471 non-diarrheal human samples, and 124 animal stool samples) were collected. A direct immunofluorescence assay (DFA) was used to detect *Cryptosporidium* spp. oocysts in concentrated stool samples by observation under a fluorescent microscope. DNA extraction, PCR amplification of the three genes (*COWP, SSU-rRNA,* and *GP60*), and sequencing analysis were performed to identify *Cryptosporidium* spp.

Results

Of 2715 samples, 15 samples (10 diarrheal samples, 2 non-diarrheal samples, and 3 animal stool samples) tested positive by PCR for the *COWP* gene. Three species of *Cryptosporidium* spp. were detected; *C. canis* (from six human diarrheal samples, two human non-diarrheal samples, and one dog sample); *C. hominis* (from four human diarrheal samples); and *C. suis* (from two pig samples). In terms of *C. hominis*, the *GP60* subtype IeA12G3T3 was detected in all four human diarrheal samples.

Conclusions

Although the number of positive samples was very small, our epidemiological data showed that the emerging pattern of each of the three species (*C. canis, C. hominis,* and *C. suis*) was different at this study site. For example, zoonotic transmission of *C. canis*, between dogs and humans was suspected. Further studies are needed to assess the risk of oocyst contamination in the wider environment, including water, in this study area.

Background

Worldwide, *Cryptosporidium* is considered an important protozoan parasite that causes gastroenteritis in a wide range of animals, including humans [1–4]. Cryptosporidiosis can lead to severe problems in immunocompromised or young hosts, in both animals and humans [5]. In the livestock farming industry, cryptosporidiosis can cause great economic losses due to weight loss and delayed growth in young animals and decreased production in adult animals [1]. In humans, infection with *Cryptosporidium* spp. can mean not only severe diarrhea but also death in patients with HIV/AIDS, patients who have received an organ transplant, and patients on immunosuppressants [6]. So far, no effective vaccines are available to prevent cryptosporidiosis in humans or in livestock [7]. Prevention and control measures are therefore essential for the

protection of vulnerable groups, such as young children and severely immunocompromised individuals, as there are only a few safe and effective therapeutic options available.

In countryside of Vietnam, where livestock live closely with humans, *Cryptosporidium* transmission to humans can easily occurs via the interaction between animals and humans [8]. People can become infected by ingesting infective *Cryptosporidium* oocysts through the fecal–oral route, including directly from infected persons (human-to-human) or animals (animal-to-human), or indirectly through the consumption of contaminated drinking water or food [1]. A distinctive pattern of transmission in the countryside of Vietnam may be environmental contamination through infected manure, which is applied to the land. It has been reported that Asia has the highest oocyst load from livestock manure compared with the loads seen on other continents [9]. One of the reasons may be climate. For example, *Cryptosporidium* oocysts can be maintained for a long time in Vietnam because the climate is warm and humid in the summertime, which is suitable for the survival of oocysts [10]. Also, Vietnam has a rainy season with heavy rainfall, which may facilitate *Cryptosporidium* oocysts spreading in the environment, leading to infections in humans and animals [11]. Previous studies conducted in Southeast Asia, including in Vietnam, have shown that there is the possibility of human *Cryptosporidium* infections originating from contaminated environmental sources [12]. To prevent and control cryptosporidiosis in agricultural areas, it is important to improve our understanding of environmental transmission routes of *Cryptosporidium*. An integrated genotyping approach would help in identifying sources of infection and routes of transmission under Vietnamese agricultural conditions [8].

Currently, many species and genotypes of *Cryptosporidium* spp. have been described, in a wide range of animals, livestock, and wild animals [2, 3, 5, 12]. It was previously thought that each species of *Cryptosporidium* had a limited host range and was able to infect only a single host or closely related host species [3]. However, according to the latest data it is likely that each species possesses a very broad range of hosts [3]. Molecular epidemiological surveillance-based studies have revealed an expanded host range of each *Cryptosporidium* spp. and their geographic distribution [13]. However, there is a scarcity of information about the molecular epidemiology of *Cryptosporidium* spp. in Vietnam. Therefore, our objective was to investigate the occurrence of *Cryptosporidium* infections and the potential for transmission of *Cryptosporidium* spp. between animals and humans in Vietnam.

Methods

Sampling (diarrheal sample collection from humans, non-diarrheal sample collection from humans, and stool sample collection from animals)

The study area (12 km^2) was a typical agricultural area in northern Vietnam, located about 75 km southeast of Hanoi. A total 2715 samples, comprising both human and animal stools, were collected using three sampling methods in Hien Khanh commune, Vu Ban district, Nam Dinh province, Vietnam. Through prospective collection for human diarrheal samples (October 2014 to March 2017), 2120 human diarrheal samples were collected from 1508 residents from 311 randomly selected households that had children of less than 5 years of age. (The detailed information is in Iwashita et al. submitted to BMC public health). Through cross-sectional collection for human non-diarrheal samples (September to October 2014), 471 human non-diarrheal samples were collected from 471 residents from 105 households, which were randomly selected from 311 households used for

diarrheal sampling (The detailed information is in Sugamoto et al. submitted to Parasite Epidemiol. Control.). Cross-sectional collection of animal stool samples (August to October 2015) yield 124 animal stool samples from animals (without clinical signs) present in this study area, such as buffalos, dairy and beef cattle, pigs, dogs, and monkey and boar (The detailed information is in Iwashita et al. submitted to Parasite Epidemiol. Control.).

Procedure of the detection of Cryptosporidium spp.

For all 2715 samples (2120 human diarrheal samples, 471 human non-diarrheal samples, and 124 animal stool samples), the formalin-ether sedimentation technique (406th Medical General Laboratory; MGL) was used to concentrate protozoal oocysts in stool samples [14]. A direct Immunofluorescence assay (DFA) using antibodies tagged with the fluorescent markers, DyLight488 (ARK Fluor Ab C/G-DyLight488, ARK Resource Co., Ltd.) was applied to detect oocysts of *Cryptosporidium* spp. in the concentrated stool samples under a fluorescent microscope (Eclipse 90i, Nikon Instruments Inc.) [14]. Using antibodies tagged with fluorescent markers, DyLight488 is able to target not only *Cryptosporidium* spp. but also *Giardia* spp. Detection by DFA was simultaneously conducted in previous study to detect *Giardia* spp. using the same 2715 samples (Iwashita et al. submitted to Parasite Epidemiol. Control.). In terms of human stool samples (2120 diarrheal samples and 471 non-diarrheal samples), any samples positive for *Cryptosporidium* spp. by DFA had their DNA extracted for PCR and sequencing analysis. All 124 animal stool samples, including those positive or negative for *Cryptosporidium* spp. by DFA, had DNA extracted for PCR and sequencing analysis.

DNA extraction

Stool samples concentrated using the MGL technique were washed twice with sterile water and then ten freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95℃ for 5 min) were performed to disrupt the oocysts. DNA was then extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, Calfolnia) following the manufacturer's instructions and stored at -20℃ prior to use. To avoid cross-contamination between animal and human samples, both samples was separately treated.

PCR analysis of the three genes (*COWP, SSU-rRNA, GP60*)

Fragment of the oocyst wall protein (*COWP*) gene [15] and the small-subunit ribosomal RNA (*SSU-rRNA)* gene [16, 17] were amplified using nested PCR protocols for the identification of *Cryptosporidium* species (Table 1). Fragments of the 60-kDa glycoprotein (*GP60*) gene was also amplified using nested-PCR protocols [18, 19] for further subtyping of *Cryptosporidium* species, especially *C. homins* and *C. parvum* (Table 1). PCR was performed using a MyCycler thermal cycler (Bio-Rad, Hercules, USA). Each 25 μL reaction mixture contained GoTaq Green Master Mix (containing Go Taq® DNA Polymerase, dNTP mixture, Green Go Taq Reaction Buffer, MgCl2; Promega) with 5% dimethyl sulfoxide (Sigma–Aldrich, USA) and 0.4 mg/ml BSA (Sigma-Aldrich, USA). Two types of nested-PCR protocols were used to prepare the *SSU-rRNA* [16, 17] and *GP60* genes [18, 19] (Table 1). All samples were amplified using three nested-PCR protocols targeting the *COWP* [15] and *SSU-rRNA* genes [16, 17] (Table 1) and all second PCR products were evaluated by a 1.5% agarose gel electrophoresis. In terms of the *COWP* gene [15], both the first and second PCR products were additionally evaluated to identify longer sequences. For all the PCR reactions, *Cryptosporidium*-positive DNA and distilled water were used as positive and negative controls, respectively.

Sequencing and phylogenetic analyses.

All positive PCR products were purified using an MonoFas DNA Purification Kit (GL Sciences, Tokyo, Japan) and sequenced in both directions (with forward and reverse primers) using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). All sequences including *SSU-rRNA*, *COWP* and *GP60* were analyzed using the BLAST program (http://blast.ncbi.nih.gov/BLAST.cgi) for homology searches. *Cryptosporidium* species reference sequences were obtained from GenBank to ensure accurate species/genotype and subtype identity, and reports of human cases were searched [20–26] (**Additional file 1: Table S1**). Phylogenetic analyses of the *COWP* and *SSU-rDNA* sequences of different *Cryptosporidium* species and genotypes were performed using MEGA (Molecular Evolutionary Genetic Analysis) 6.0 software (http://www.megasoftware.net/).

Results

A total of 2591 human stool samples (2120 human diarrheal samples and 471 human non-diarrheal samples) were screened with DFA. Of these, 77 human diarrheal samples and 11 human non-diarrheal samples were positive for *Cryptosporidium* spp. DNA was extracted from these samples for PCR analysis. All 124 animal stool samples, regardless of whether they were positive or negative for *Cryptosporidium* spp. by DFA had DNA extracted for PCR analysis (Fig. 1).

Through our sampling methods, only fifteen samples (ten human diarrheal samples, two human non-diarrheal samples, and three animal stool samples) were found to be positive for *Cryptosporidium* spp. by PCR (Table 2). The patterns of *Cryptosporidium* infection caused by *C. canis*, *C. hominis* and *C. suis* at our study site became clear. *Cryptosporidium canis* was detected from human non-diarrheal (Fig. 2) and diarrheal samples (Fig. 3), and dog stool samples (Fig. 4□), while *C. suis* was only detected in pigs (Fig. 4□). *Cryptosporidium hominis* was only detected in human diarrheal samples (Fig. 3). Through our prospective diarrhea sampling, four samples that were positive for *C. homins* were coincidentally detected from two neighboring households at the same time (Fig. 3□). The six samples positive for *C. canis*, on the other hand, were sporadically detected without any obvious patterns of occurrence. The *C. hominis*-positive cases were aged 11, 15, 37 and 70 years (Table 2). There were no samples from children aged less than 5 years, the group most vulnerable to severe diarrhea. On the other hands, the age of *C. canis*-positive cases were 1, 2, 5, 6, 7, and 26 years for diarrheal samples and 2 years for the two non-diarrheal samples (Table 2). No samples from buffalos or dairy and beef cattle were positive for *Cryptosporidium* spp. Primers targeting the *COWP* gene had a higher detection rate (15/15) than primers targeting the *SSU-rRNA* gene (9/15) (Table 2). Samples in which *C. canis* and *C. suis* were identified by PCR using the *COWP* gene and/or the *SSU-rRNA* gene, failed to amplify any DNA using primers targeting the *GP60* gene, because the *GP60* primers were more specific for *C. hominis*. In terms of the *COWP* and *SSU-rRNA* genes, the phylogenetic tree constructed using MEGA software is shown in Figs. 5 and 6. Bootstrap values were obtained using 1000 pseudo-replicates; those > 50% are shown on nodes. The evolutionary distances were computed using the Kimura-2 parameter method [27]. GenBank accession numbers are shown before the species names in Figs. 5 and 6 and Additional file 1: Table S1.

Molecular diagnosis using the COWP gene

Good quality sequencing data of *COWP* gene were available for the 15 isolates. Phylogenetic analysis could identify three species of *Cryptosporidium* spp. (Fig. 5). Two isolates (from pig stool samples) assigned to *C.*

suis, exhibited 100% identity with the reference sequence AF266270.1, between position 359 to 712. Four isolates (four human diarrheal samples) assigned to the *C. hominis*, exhibited 100% identity with the reference sequence AF266265.1, while three isolates comprised 506 bp between positions 359 to 864 and one isolate (CDS_1788) comprised 354 bp between positions 359 to 712. Nine isolates (one dog sample, six human diarrheal samples, and two human non-diarrheal samples) were assigned to *C. canis*. One of them exhibited 100% identity with the reference sequence AF266274.1, between 17 to 370 (Ani_140). The remaining eight isolates differed by one or two single nucleotide polymorphisms (SNPs) with it, although the amplified product sizes were different: CDS_263 and CDS_449 were 354 bp, while the others are 506 bp (Table 3). The unique sequences described here have been deposited in the GenBank database under accession numbers LC503969 to LC503972 (Table 3). Details of the results using *COWP* gene are shown in Additional file 2: Table S2.

Molecular diagnosis using the SSU-rRNA gene

Good-quality sequencing data of *SSU-rRNA* gene were available for the nine isolates. Phylogenetic analysis could clearly distinguish three species of *Cryptosporidium* spp., the same as the analysis based on the *COWP* gene (Fig. 5, 6). Two isolates (from pig stool samples) assigned to *C. suis* exhibited 100% identity with the reference sequence AB449824, between position 98 to 638. Three isolates (three human diarrheal samples) assigned to the *C. hominis* exhibited 100% identity with the reference sequence AF108865.1, while two isolates comprised 543 bp between positions 313 to 855 and one isolate (CDS_1788) comprised 735 bp between 241 to 975. Four isolates (one dog sample, two human non-diarrheal samples, and one human diarrheal sample) assigned to *C. canis* showed 100% identity with the reference sequence AB210854.1, between 312 to 846 (535 bp). Details of the results using the *SSU-rRNA* gene are shown in Additional file 3: Table S3.

Molecular diagnosis using the GP60 gene

In this study, all four *C. hominis*-positive samples using the *COWP* and/or *SSU-rRNA* genes were successfully subtyped using the *GP60* gene (Table 2). One subtype from all four isolates, IeA12G3T3 was identified according to the scheme described by Xiao [28]. Other *Cryptosporidium*-positive samples using *COWP* and/or *SSU-rRNA* gene were not amplified with primers for the *GP60* gene. Details of the results using the *GP60* gene are shown in Additional file 4: Table S4.

Discussion

Although *Cryptosporidium* infection was not highly prevalent among human or animal samples tested from this study site, the potential for zoonotic transmission of *C. canis* from dogs to humans and vice versa was apparent. We found no positive samples in cattle or buffalos we tested, although zoonotic transmission from these species has been reported by researches in other areas studied, such as China [29]. Instead, in our study area, there were samples positive for *Cryptosporidium* in dogs, pigs and humans. Although most cases of cryptosporidiosis caused by *C. canis* globally have been reported in dogs, some cases have been reported in humans, according to other studies and our results [30, 31]. For example, in Siem Reap, northwest Cambodia, human stool samples collected from five patients attending the out-patient clinic or admitted to hospital were positive for *C. canis* [32]. The dogs at this study site were free roaming, as is commonly the case in the countryside of Vietnam, and had close relationship with humans. The infection may occur either inside or

outside of households. Human and animal feces can easily contaminate the environment, including with *Cryptosporidium* spp., due to a lack of a sewage system at the study site.

In previous studies conducted in Vietnam and surrounding countries, *Cryptosporidium* infections have been identified in animals and/or humans using methods such as microscopic analysis, immunofluorescence, enzyme-linked immunosorbent assays, and PCR [32, 33, 42–50, 34–41]. Most studies applied molecular methods to identify species and genotypes of *Cryptosporidium* (Table 4). In Vietnam and surrounding countries or areas (e.g., Laos, Cambodia, the southern provinces of China), the following species of *Cryptosporidium* were detected (with host animals in parentheses): *C. andersoni* (dairy cattle) [46]; *C. bovis* (beef cattle) [33]; *C. canis* (human) [32]; *C. hominis* (human, monkey) [32, 47]; *C. meleagridis* (human) [32]; *C. muris* (monkey) [47]; *C. parvum* (human) [32]; *C. ryanae* (cattle) [33]; *C. scrofarum* (pig) [49]; *C. suis* (pig, human, environmental sample) [32, 35, 38]; *C. ubiquitum* (human) [32]; *Cryptosporidium* avian genotype II (ostrich) [37]; *Cryptosporidium* bat genotype (bat) [45]; and *Cryptosporidium* pig genotype II (pig) [35] (Table 4). In Cambodia, Moore et al. detected many species of *Cryptosporidium* from symptomatic children in a hospital [32]. The species reported in their study were *C. canis, C. hominis, C. meleagridis, C. parvum*, and *C. suis*, all of which have zoonotic potential [31]. *Cryptosporidium hominis* and *C. canis* were detected in human samples collected at our study site, but *C. suis* was not. *Cryptosporidium canis* and *C. suis* were detected in animal samples collected at our study site, but *C. hominis* was not. All species detected in this study area could easily contaminate the environment and/or water and/or food through the excretion of stools that contain oocysts, and there is always a risk of a spillover of disease transmission to humans. Table 4 gave us the opportunity to estimate the potential for transmissions between animals and humans by different species of *Cryptosporidium* in Vietnam and surrounding countries. It should be noted that the majority of patients with HIV in the studies listed in Table 4 were infected with *C. hominis* and *C. parvum*. This is consistent with a previous study of children infected with HIV in Kenya [51, 52]. Although *C. hominis* is widely considered to be a human-specific *Cryptosporidium* species, it has increasingly been reported in animals from all over the world except Vietnam [53–55].

Cryptosporidium hominis is known to cause severe to moderate infections in humans [56]. More than ten subtypes of *C. hominis* have been identified, based on sequence analysis of the *GP60* gene [30]. It has been reported that *GP60* analysis has discriminatory power to determine transmission dynamics and source of infection [31].

In our study, only one subtype, IeA12G3T3, was detected from all human diarrheal samples identified as having *C. hominis* based on *COWP* and/or *SSU-rRNA* gene analysis. This IeA12G3T3 subtype has also been identified in various samples in other countries, such as stool specimens from individuals with HIV infection in Jamaica [57], water samples in Shanghai, China [58], stool specimens from Filipino immigrants in Qatar [59] and stool specimens from an immunocompromised patient in Slovakia [60]. Fortunately, this subtype has never been found to be responsible for an outbreak, and its infectivity or degree of virulence have yet to be reported. This is different from a notorious subtype, such as IbA10G2, which is widely distributed and was responsible for outbreaks in Europe, Australia, and the USA [31]. Following our investigation using *GP60* subtyping for *C. hominis*, it became clear that one subtype (IeA12G3T3) was dominant at our study site. We suspect that the transmission route might have been the same in all cases, occurring in neighboring households.

Our study has several limitations. First, the number of positive *Cryptosporidium* spp. cases was very small, comprising ten human diarrheal samples, two human non-diarrheal samples, and three animal stool samples.

As the different sampling methods and timing for collecting these three kinds of samples could not be perfectly matched, it was very difficult to accomplish our ambitious goal to determine transmission routes using these samples.

Second, we are concerned that the PCR analyses used in our study might have underestimated the prevalence of *Cryptosporidium* spp. This could be due to one or more of the following reasons: DNA degradation [61], insufficient DNA [37], inhibition of PCR [62], and prolonged storage of the stool samples [63]. We were also concerned about cross-reactions with non-target organisms, such as algae, when we apply DFA [64], meaning we could not count the number of oocysts. False positive microscopy results were also not ruled out. Although our study objectives did not include estimating the exact prevalence or incident of cryptosporidiosis, such low detection of *Cryptosporidium* spp, was likely to miss several transmission routes and affect the objective of this study. In terms of storage of the samples, it was difficult to exactly match the conditions of storage time until DNA extraction for all samples collected over three years. As we followed the experimental process described in Fig. 1, the time until DNA extraction varied depending on the time to complete detection by DFA using microscopy, which was time consuming and laborious. In fact, all our stool samples were stored in the suitable condition for prolonged storage, according to the conditions noted by Jongwutiwes et al. [65]. In addition, each primer has a different detection rate when identifying *Cryptosporidium* spp. The primers targeting the *COWP* gene have higher detection rates than the primers targeting the *SSU-rRNA* region. The *GP60* gene is usually used as a marker for the detection of *C. parvum* and *C. hominis* [28]. Therefore, we did not succeed using PCR to identify *GP60* for the samples already detected as *C. suis* and *C. canis* by *COWP* and *RRU-rRNA*. Moreover, we did not detect any *C. parvum*-positive samples. Generally, in agricultural area, the risk of infection may be greater for larger livestock, such as buffalos and cattle, which are the main hosts of *C. parvum*. We suspected that zoonotic transmission was occurring from cattle and buffalos to humans. However, false negative PCR result was not ruled out, as mentioned above. In agricultural area of Vietnam, where there are many free-roaming livestock and no sewage systems, water is considered to be an important mechanism in the transmission of *Cryptosporidium* spp. If contamination of water from a particular population of animals is suspected, investigation of the water itself is needed to verify the risk of *Cryptosporidium* spp. to public health. In fact, contamination of water with *Cryptosporidium* spp. from pig farms at our study site might represent a growing problem unless sanitary conditions are improved.

Third, it is very difficult to determine the pathogenicity and virulence of *Cryptosporidium*. In terms of human cases at our study site, we suspect that *C. hominis* was more pathogenic than *C. canis*, even though we could not address this risk through our study design. Four cases of *C. hominis*, of the same subtype, were detected at the same time from human diarrheal samples. On the other hand, *C. canis* were detected sporadically from many kinds of samples, such as human diarrheal and non-diarrheal samples and dog stool samples, which were spread throughout our study site. The pattern of *C. hominis* and *C. canis* throughout our study were quite different and we suspect that *Cryptosporidium* spp. virulence or pathogenicity also differs. There have been reports of differences in clinical manifestations among *Cryptosporidium* species and subtypes [66–71]. In adults and children infected with HIV, for example, it has been suggested that *C. hominis* is mainly associated with diarrhea, nausea, vomiting, and malaise, whereas *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis* are associated with diarrhea only [66, 67]. Another report, of the medium-to long-term impact of cryptosporidiosis, suggested that *C. hominis* infection is mainly associated with fatigue and abdominal pain greater than that seen with *C. parvum* infection [69, 70]. In addition, different subtypes of *C. hominis* have been linked to variable

clinical outcomes [71]. However, the etiology of diarrhea itself is very complicated, and we could not rule out pathogens except *Cryptosporidium* spp. as the cause of diarrhea. In fact, there was some additional information about other diarrheal pathogens from the human stool samples used in this study. These other pathogens were as follows: rotavirus, norovirus GI and norovirus GII, *Aeromonas* spp., *Campylobacter* spp., *Clostridium difficile*, Enterotoxigenic *Escherichia coli*, enteroaggregative *E coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Giardia* spp., *Entamoeba histolytica*. The presence of these pathogens was investigated using the same samples already detected *Cryptosporidium* spp. in this study. Coincidentally, four samples which were positive for *C. hominis* were also positive for enteroaggregative *E coli* (unpublished data). In cases of mixed infection with various diarrheal pathogens, it is very difficult to identify the etiology of diarrhea.

To control cryptosporidiosis, a “One Health approach” can be applied; this is a collaborative approach among public and veterinary health professionals. Although the distribution patterns of *Cryptosporidium* spp. vary from country to country and even from one region to another, we believe that even our small quantity of local molecular epidemiological data could contribute to improve the knowledge around the transmission of this parasite. In Vietnam, there have still been few studies focused on both public and veterinary health, as was the case with our study. Although our results are limited to one small area, our information could form part of a network of molecular-based surveillance systems. We believe that the accumulation of each local reports has a possibility to help reduce disease incidence in country level.

Conclusions

As cryptosporidiosis takes the largest toll on the health of vulnerable populations, such as patients who are immunodeficient and young children living in low-income settings, it is important to prevent infection by strictly minimizing the number of oocysts in the environment. Although the detection rate of *Cryptosporidium* spp. in our study was not high, it was certain that at least three species of *Cryptosporidium* were present. Particularly with *C. canis*, zoonotic transmission between dogs and humans was suspected. *Cryptosporidium* spp. is an environmentally ubiquitous protozoan parasite. Our study only used stool samples only, although *Cryptosporidium* spp. can also be found in water and environmental samples. As our study site is a typical agricultural area where there are many free-roaming livestock and no sewage systems, environmental surveillance would be helpful in avoiding outbreaks of cryptosporidiosis. Not only animal stools but also human stools could contaminate water with *Cryptosporidium* spp. at our study site, due to no sewage systems.

Abbreviations

COWP: oocyst wall protein

DFA: direct Immunofluorescence Assay

GP60: 60-kDa glycoprotein

MEGA: Molecular Evolutionary Genetic Analysis

MGL technique: formalin-Ether sedimentation (406th Medical General Laboratory; MGL) technique

SNP: single nucleotide polymorphisms

SSU-rRNA: small-subunit ribosomal RNA

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the Graduate School of International Health and Development, Nagasaki University and the Institutional Review Board of NIHE in Vietnam. Written informed consent was obtained from participants, who were the head of the household for each household. A verbal consent statement was obtained from livestock owners prior to the collection of fecal samples from their private land.

As a major consideration, no patients who were immunocompromised or had AIDS/HIV were included in the study. Although the participants were notified and understood that the test results of their samples would not be available to assist in any treatment, they would be monitored for clinical manifestations of *Cryptosporidium* infection and other diarrheal symptoms trained health workers. The participants were advised to report any abnormal health status to the local physician in the Hien Khanh Commune Health Station, which was staffed with three physicians, one pharmacist, one midwife, and one nurse. If any manifestations of cryptosporidiosis occurred, all participants with diarrhea were treated at the community health center, according to Ministry of Health guidelines. In cases of severe diarrhea, participants were referred to the district or the provincial hospital for appropriate laboratory testing and treatment.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HI, TT, AT and TS conceived and designed this study. VDT, NHT and PDT planned the study in Vietnam. HI, AT, TS, NHT, PDT, PHQA, HTD and LNT collected the field data, and HI, TT, AT, TS, PHQA, HTD and LNT conducted the laboratory work. HI drafted the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1. Primers used to detect and to sequence *Cryptosporidium* spp.

Target gene	Step	Primer name	Nucleotide sequence (5' to 3')	Polarity	Product (bp)	Cycle condition	Reference
COWP	1st PCR	Cry-15	GTAGATAATGGAAGAGATTGTG	forward	550 bp	95°C, 5 minutes, 1 cycle; 94°C, 30 seconds, 55°C, 30 seconds, 72°C, 1 minute, 45 cycles; 72°C, 7 minutes, 1 cycle	Yu et al. (2009)
		Cry-9	GGACTGAAATACAGGCATTATCTTG	reverse			
	2nd PCR	Cry-15	GTAGATAATGGAAGAGATTGTG	forward	311 bp		
		cowpnest-R2	TCTGTATATCCWGGTGGGC	reverse			
SSUrRNA	1st PCR	18SiCF2	GACATATCATTCAAGTTTCTGACC	forward	758-763 bp	94°C, 5 minutes, 1 cycle; 94°C, 30 seconds, 58°C, 30 seconds, 72°C, 20 seconds, 45 cycles; 72°C, 10 minutes, 1 cycle	Ryan et al. (2003)
		18SiCR2	CTGAAGGAGTAAGGAACAACC	reverse			
	2nd PCR	18SiCF1	CCTATCAGCTTTAGACGGTAGG	forward	585-590 bp		
		18SiCR1	TCTAAGAATTTCACCTCTGACTG	reverse			
SSUrRNA	1st PCR	Cry-SSU-F2	TTCTAGAGCTAATACATGCG	forward	1325 bp	95°C, 5 minutes, 1 cycle; 95°C, 45 seconds, 55°C, 45 seconds, 72°C, 1 minite, 45 cycles; 72°C, 7 minutes, 1 cycle	Xio et al. (1999)
		Cry-SSU-R2	CCCATTTCCTTCGAAACAGGA	reverse			
	2nd PCR	Cry-SSU-F3	GGAAGGGTTGTATTTATTAGATAAAG	forward	825- 830 bp		
		Cry-SSU-R3	AAGGAGTAAGGAACAACCTCCA	reverse			
GP60	1st PCR	AL3531	ATAGTCTCCGCTGTATTC	forward	~850 bp	94°C, 5 minites, 1 cycle; 94°C, 45 seconds, 54°C, 45 seconds, 72°C, 90 seconds, 45 cycles; 72°C, 10 minutes, 1 cycle	Sulaiman et al. (2005) Gatei et al. (2006)
		AL3533	GAGATATATCTTGGTGCG	reverse			
	2nd PCR	AL3532	TCCGCTGTATTCTCAGCC	forward	~550 bp	95°C, 5 minutes, 1 cycle; 94°C, 45 seconds, 55°C, 45 seconds, 72°C, 90 seconds, 45 cycles; 72°C, 10 minutes, 1 cycle	
		AL3533	GAGATATATCTTGGTGCG	reverse			
GP60	1st PCR	AL3531	Same as above	forward	~850 bp	Same as above	Sulaiman et al. (2005) Gatei et al. (2006)
		AL3533	Same as above	reverse			
	2nd PCR	AL3532	TCCGCTGTATTCTCAGCC	forward	400-500 bp	95°C, 5 minutes, 1 cycle; 94°C, 45 seconds, 54°C, 45 seconds, 72°C, 1 minutes, 45 cycles; 72°C, 10 minutes, 1 cycle	
		LX0029	CGAACCACATTACAAATGAAGT	reverse			

Table 2. Genotype characterization of *Cryptosporidium* isolates from stool samples

Sample ID	ID	Sample	Age (years old)	Sampling period	Genotype		
					COWP	*SSU-rRNA*	*GP60*
	589_7	Human non-diarrheal	2	28 October, 2014	*C. canis*	*C. canis*	
	1766_4	Human non-diarrheal	2	19 September, 2014	*C. canis*	*C. canis*	
Ani_74		Piglets[1]	young[1]	4 September, 2015	*C. suis*	*C. suis*	
Ani_97		Sow & Piglet[2]	adult+young[2]	22 September, 2015	*C. suis*	*C. suis*	
Ani_140		Dog[3]	adult[3]	September, 2015	*C. canis*	*C. canis*	
CDS_263	1140_6	Human diarrheal	2	26 Aptil, 2015	*C. canis*		
CDS_449	551_5	Human diarrheal	26	8 July, 2015	*C.canis*		
CDS_1147	1032_3	Human diarrheal	7	19 March, 2016	*C. canis*		
CDS_1197	242_6	Human diarrheal	1	17 April, 2016	*C. canis*	*C. canis*	
CDS_1216	1731_8	Human diarrheal	6	21 April, 2016	*C. canis*		
CDS_1284	122_6	Human diarrheal	5	11 May, 2016	*C. canis*		
CDS_1788	400_2	Human diarrheal	70	14 October, 2016	*C. hominis*	*C. hominis*	*C. hominis*[4]
CDS_1789	400_4	Human diarrheal	37		*C. hominis*	*C. hominis*	*C. hominis*[4]
CDS_1790	422_3	Human diarrheal	15		*C. hominis*	*C. hominis*	*C. hominis*[4]
CDS_1791	422_4	Human diarrheal	11		*C. hominis*		*C. hominis*[4]

[1] This was mixed stool sample collected from 12 piglets. [2] This was mixed stool sample collected from 1 sow and 1 piglet.

[3] This was collected from one adult dog kept in the household. [4] Subtype IeA12G3T3 was identified.

Table 3. Diversity and frequency of all sequence results including single-nucleotide polymorphisms of *C. canis* isolate at the *COWP* gene

Sample ID	Sample	GenBank accession number	Product size (bp)	Stretch	Nucleotide at position of reference sequence AF266274.1			
					213	320	352	372
					A	C	A	T
589_7	Human non-dirrheal	LC503972*	506	17-522				C
1766_4	Human non-dirrheal	LC503972*	506	17-522				C
Ani_140	Dog	-	354	17-370				
CDS_263	Human diarrheal	LC503969*	354	17-370			G	
CDS_449	Human diarrheal	LC503970*	354	17-370		T		
CDS_1147	Human diarrheal	LC503971*	506	17-522	G			C
CDS_1197	Human diarrheal	LC503972*	506	17-522				C
CDS_1216	Human diarrheal	LC503972*	506	17-522				C
CDS_1284	Human diarrheal	LC503972*	506	17-522				C

* The novel sequences without heterogeneous positions were newly submitted to GenBank

Table 4. Studies of Cryptosporidium infection in Vietnam and surrounding countries or area (Laos, Cambodia, southern provinces of China).

Country	Study area	Population/Source	Method for Prevalence calculation	Prevalence(%)	Genes for species identification	Species	Gp60 subtype (*C.hominis* and *C.parvum*)	Reference (published year) [Sampling date]
Vietnam (central)	Dac Lac province (beef farms)	native beef calves 2-6 months old	modified Ziehl–Neelsen (mZN) staining method	The overall prevalence : 18.9 % (44/232), herd level : 50 % (20/40)	18S rRNA	*C. ryanae*, *C. bovis*	NA	Nguyen et al. (2012a) [January and April 2011]
Vietnam (south)	Ca Mau province (nursery)	Asian seabass (Lates calcarifer) : formalin fixed Vietnamese L.calcarifer tissue samples	Partial 18S rRNA gene sequences	20% (2/10)	18S rRNA	*C. parvum*-like, *C. hominis*-like	NA	Gibson-Kueh et al. (2011) [2008]
Vietnam (central)	Dac Lac province (pig farm)	pig	modified Ziehl–Neelsen (mZN) staining method	14.5% (28/193)	18S rRNA, HSP70	*C. suis*, *Cryptosporidium* pig genotype II	NA	Nguyen et al. (2013a) [October 2009 to January 2010]
Vietnam (central)	BinhDinh province, KhanhHoa province and DacLac province (Farm)	pig	modified Ziehl–Neelsen (mZN) staining method	18.1% (134/740)	NA	*C. parvum**	NA	Nguyen et al. (2012b) [February and December 2009]
Vietnam (central)	Khanh Hoa province (ostrich farm)	ostrich	modified Ziehl–Neelsen (mZN) staining method	23.7% (110/464)	18S rRNA, HSP70, actin	*Cryptosporidium* avian genotype II	NA	Nguyen et al. (2013b) [January to March 2011]
Vietnam (north)	Hanam province	134 enviommantal samples	All samples positive by Immunofluorescence Assay (IFA) microscopy were screened for the presence of Cryptosporidium by PCR.	Total 35.0% (47/134); Nhue river water 41.6%(10/24), Sewage 66.7% (16 /24), Fishpond water 25.0% (8/32), Canal water 39.1% (9/23), Vegetables 15.3% (4/26), Composed water 0% (0/5)	18S rRNA	*C. suis*	NA	Nguyne et al. (2016) [February 2009 and July 2009]
Vietnam	NA	HIV-infected patients	NA	NA	18S rRNA	*C. parvum* human	NA	Gatei et al. (2003) [NA]
Vietnam	NA	2 human (children, diarrheic) samples	NA	NA	18S rRNA	*C. hominis*	NA	Ghaffari et al. (2012) [NA]
Vietnam (south)	Ho Chi Minh City, Dong Thap province, Dak Lak province, Khanh Hoa province, Thua Thien Hue province	Stool samples collected from people admitted to the hospital for diarrheal disease throughout Vietnam.	The Luminex xTAG gastrointestinal pathogen panel assay (GPP) (Luminex Molecular Diagnostics, Austin, TX, USA)	17% (83/479)	NA	NA	NA	Duong et al. (2016) [during 2009 to 2014]
Laos	Vientiane (Vientiane Capital) and Savannakhet province (Southern part of Laos)	HIV-infected patients: two public hospitals (Vientian and Savannakhet)	modified Ziehl–Neelsen (mZN) staining method	6.6% (9/137) (= Vientiane: 10.5% (7/67), Savannakhet: 2.9% (2/70))	NA	NA	NA	Paboriboune et al. (2014) [October 2009 to September 2010]
Cambodia	Phnom Penh (Cambodian Capital)	HIV-infected patients (adults): hospital	modified Ziehl–Neelsen (mZN) staining method	Patients with chronic diarrhea: 40% (16/40), Patients without chronic diarrhea: 50% (20/40)	18S rRNA	*C. hominis* *C. parvum*	NA	Chhin et al. (2006) [From January 2001 to April 2001]

*The size and morphology of all oocysts were similar to those described for *C. parvum* and related *Cryptosporidium* species and genotypes.

Table 4. Studies of Cryptosporidium infection in Vietnam and surrounding countries or area. (continued)

Contry	Study area	Population/Source	Method for Prevalence calculation	Prevalence (%)	Genes for species identification	Species	Gp60 subtype (*C.hominis* and *C.parvum*)	Reference (published year) [Sampling date]
Cambodia (North-Wstem)	Siem Reap	Children attending the out-patient clinic or admitted to hospital	Ziehl-Neelsen (mZN) staining method, 18S rRNA real time PCR	*Cryptosporidium* oocysts detected using microscopy: 2.2% (11/498) detected with molecular tests: 7.7% (38/498)	18S rRNA, Gp60	*C. hominis*, *C. meleagridis*, *C. parvum*, *C. canis*, *C. suis*, *C. ubiquitum*	*C. hominis* sybtype IaA16R6, *C. parvum* subtype IIeA7G1	Moore et al. (2016) [between 3rd April 2012 and 29th June 2012]
Cambodia	Boeng Cheung Ek lake (located 5–7 km south of the capital Phnom Penh)	water spinach and lake water	fluorescence microscope using fluorescent monoclonal antibodies	*Cryptosporidium* oocysts from water spinach samples: 17% (6/36)	NA	NA	NA	Anh et al. (2007) [from July 2004 to May 2005]
China (south)	Yunnan Province	A total of 247 bats	nested PCR based on the 18S rRNA gene	7.7% (19/247)	18S rRNA	*Cryptosporidium* bat genotype I *Cryptosporidium* bat genotype II	NA	Wang et al. (2013) [During 2010– 2011]
China (south)	Guangxi	Dairy cattle	NA	NA	18S rRNA	*C. andersoni*	NA	Wang et al. (2012) [NA]
China (south)	three monkey farms and one nature reserve in Guangxi Zhuang Autonomous Region, one zoo and one research facility in Yunnan Province	Fresh faecal specimens from non-human primates: in Guangxi Zhuang Autonomous Region (n=1079), in Yunnan Province (n=144)	nested PCR based on the 18S rRNA gene	Guangxi: 1% (11/1079) Yunnan: 0% (0/144)	18S rRNA, HSP70, GP60	*C. hominis*, *C.muris*	*C. hominis* subtype IbA12G3, IiA17	Karim et al. (2014) [2006 and 2013]
China (south)	Guangxi (commercial animal facility)	205 crab-eating macaques (*Macaca fascicularis*)→Group 1 (young monkeys in single cages, n=168), Group 2 (young monkeys in group cages, n=18), Group 3 (adults in group cases, n=19)	PCR-RFLP (18S rRNA)	Group 1: 0 % (0/168), Group 2: 0% (0/18),5.3% (1/19)	18S rRNA, Gp60	*C. hominis*	*C. hominis* subtype IdA14	Ye et al. (2014) [in December 2011]
China (south)	Yunnan province (Pig farms)	pig (n=200)	nested PCR	23% (46/200)	18S rRNA	*C. scrofarum*	NA	Zou et al. (2017) [2016]
China (south)	Guangxi Zhuang Autonomous Region	285 HIV/AID cases 150 HIV negative persons	modified acid-fast staining and nested PCR based on 18S rRNA	HIV/AIDS patients: 0.7% (2/285), HIV negative persons: 0% (0/150)	18S rRNA	*C. andersoni*, *C. hominis*	NA	Zon-Fu et al. (2016) [2016]

NA: Not applicable

Figures

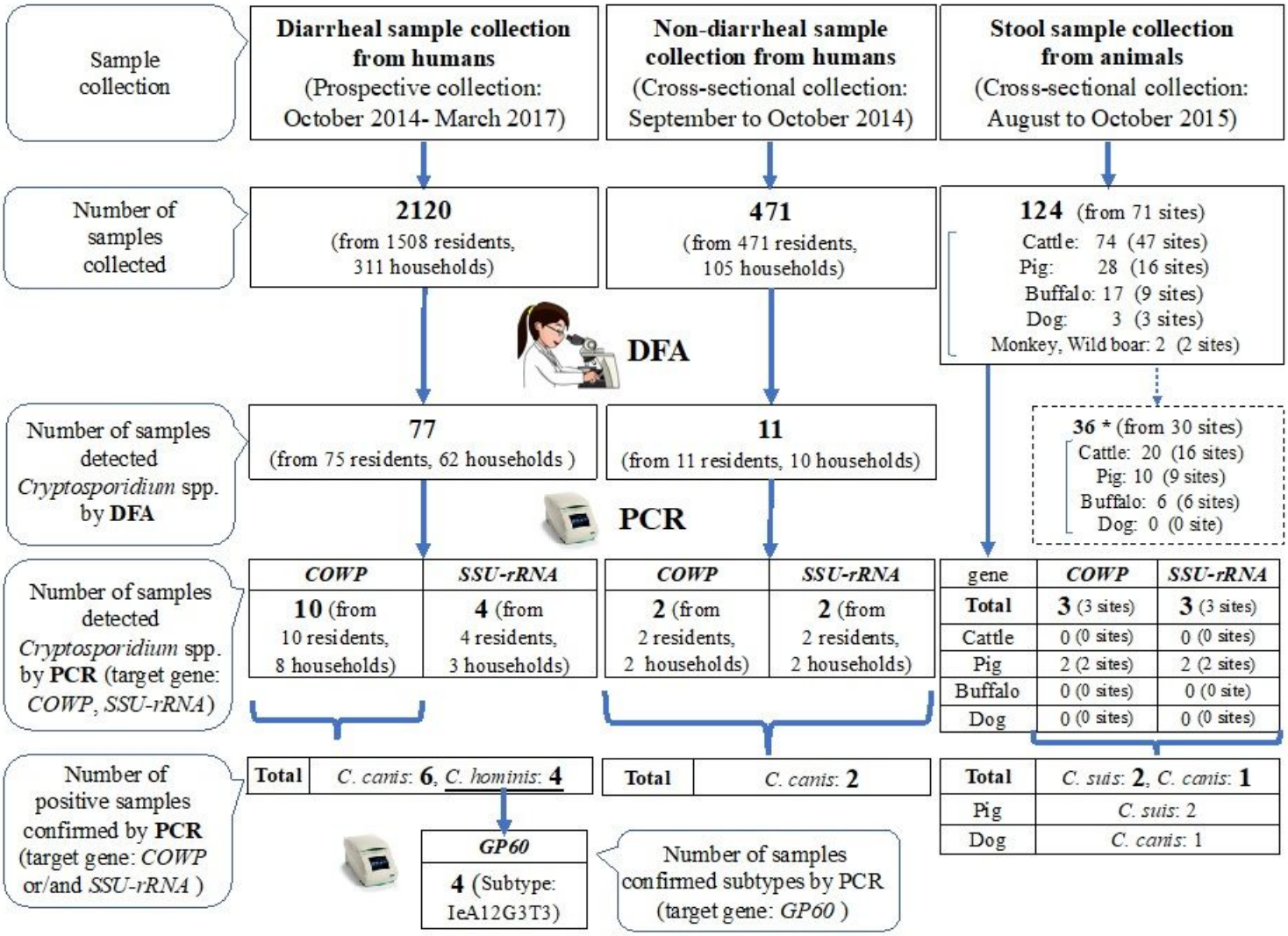
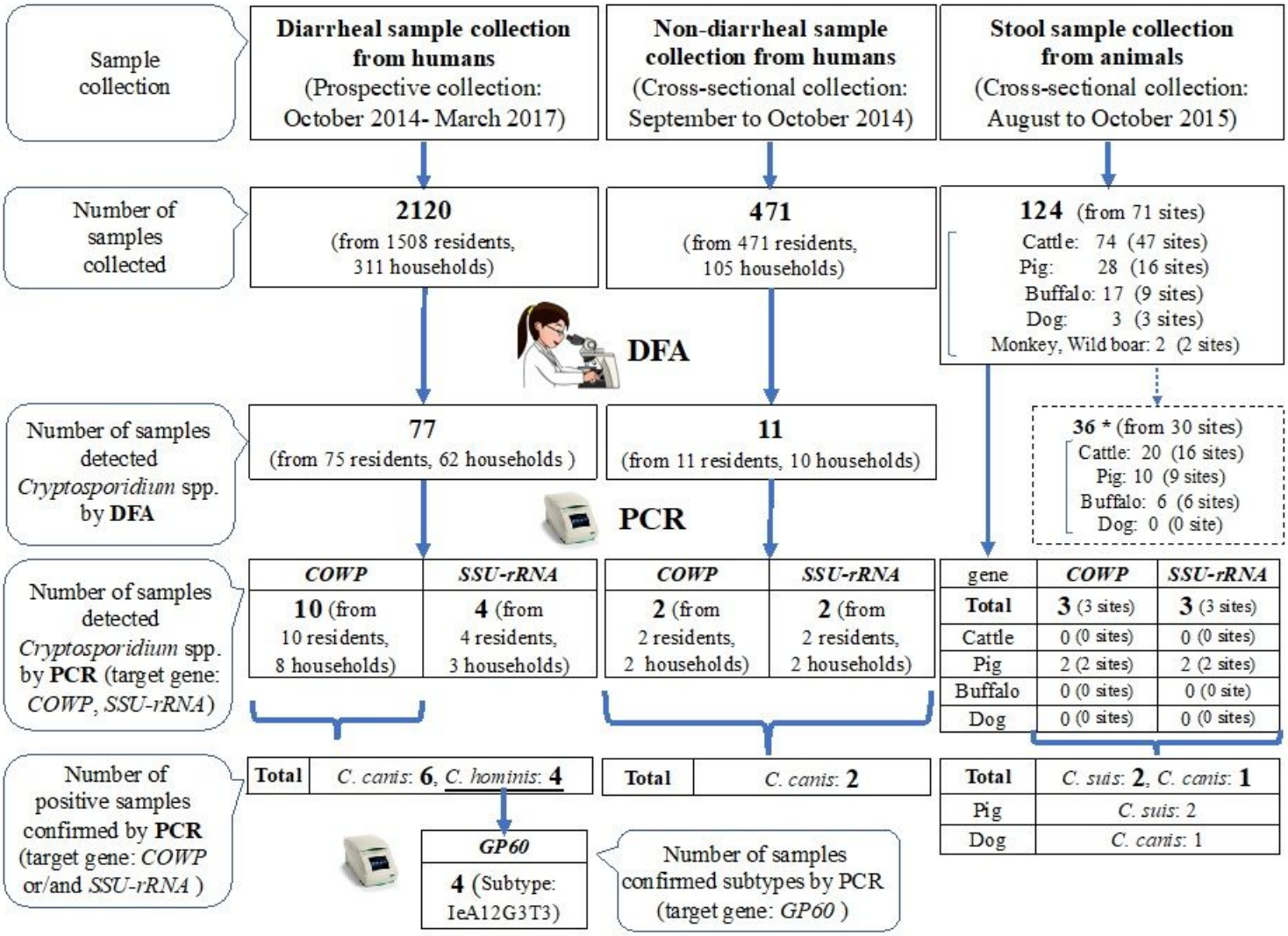


Figure 1

Algorithm for diagnosis and results of molecular genotyping of Cryptosporidium spp.



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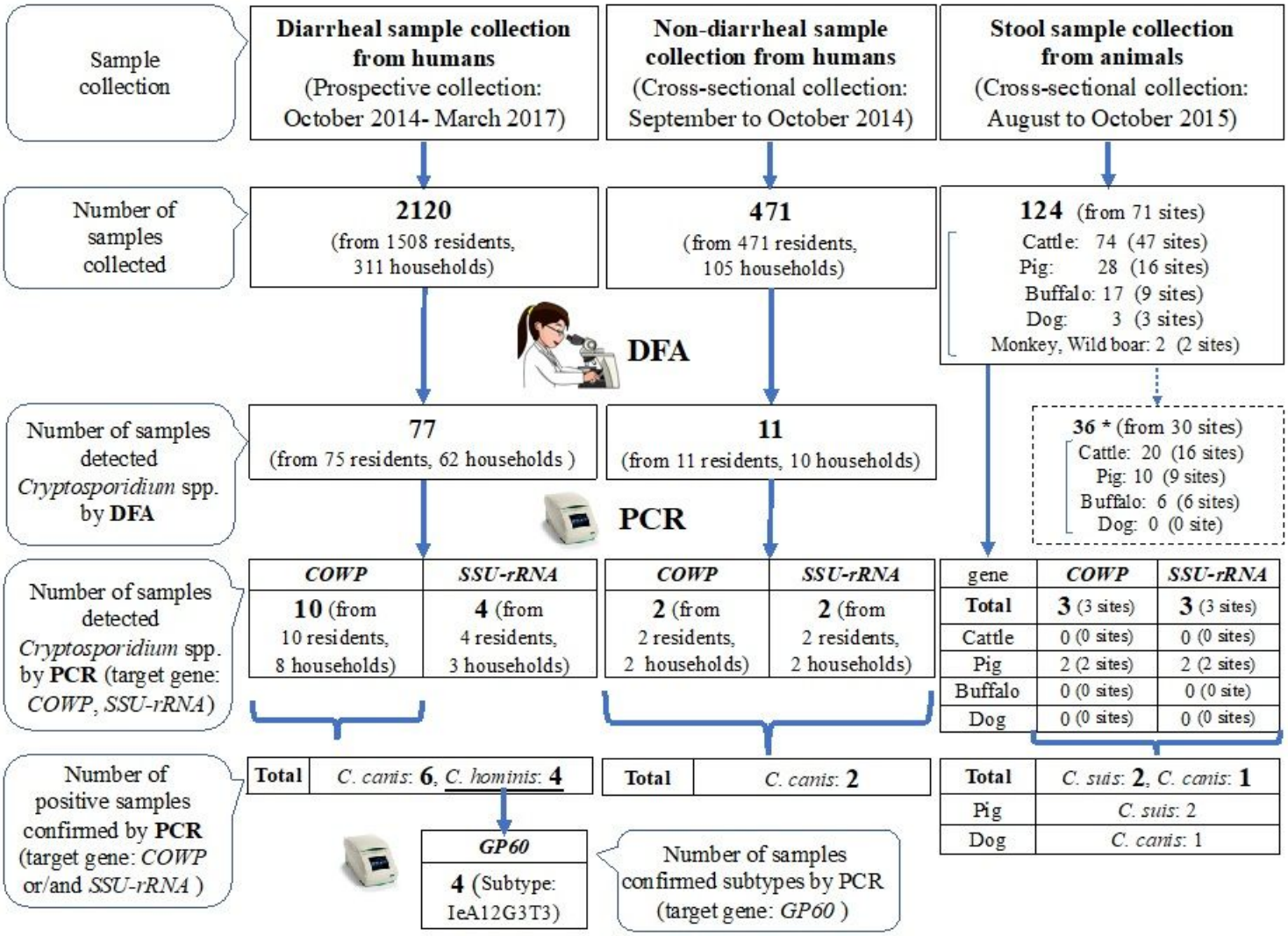

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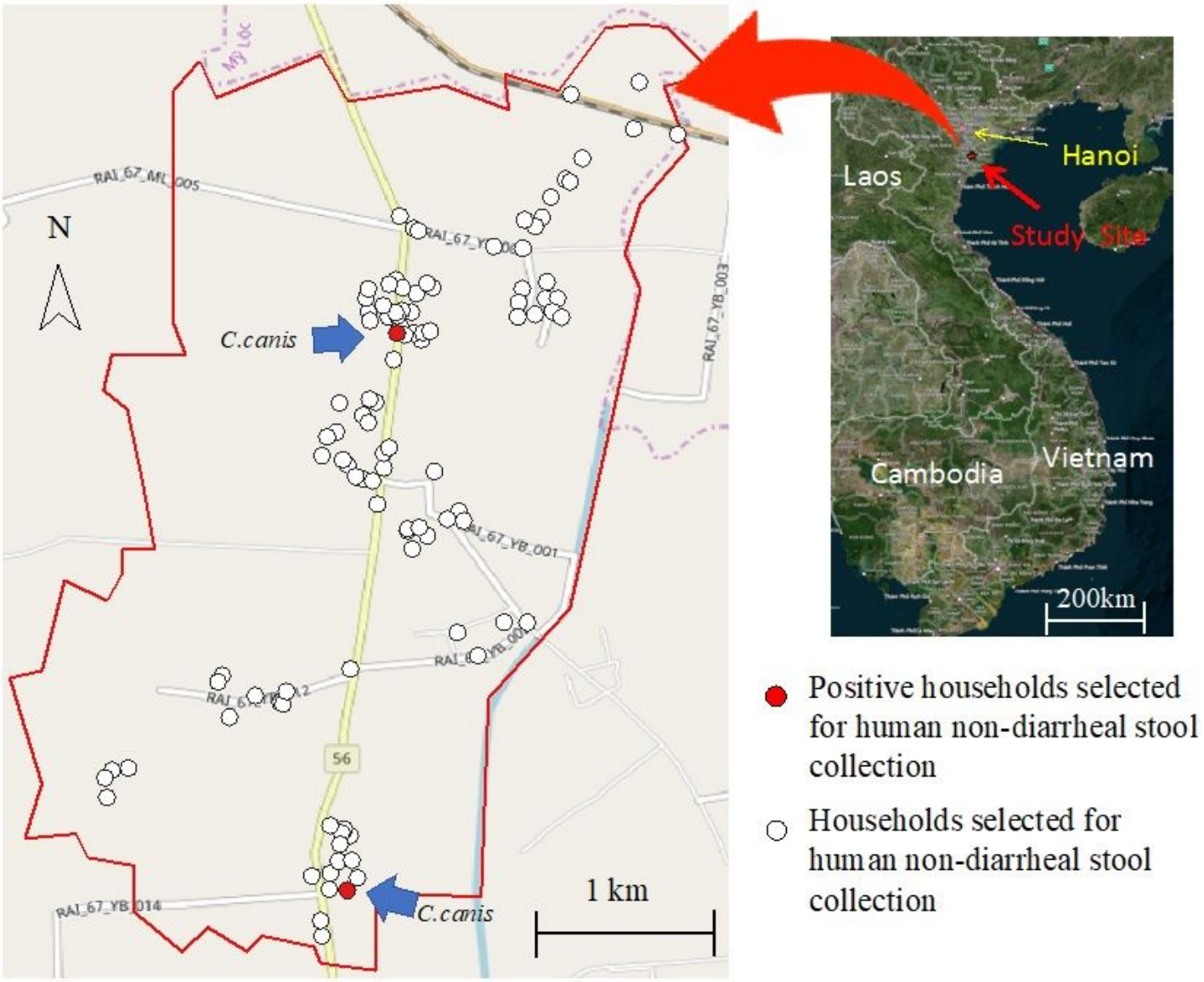
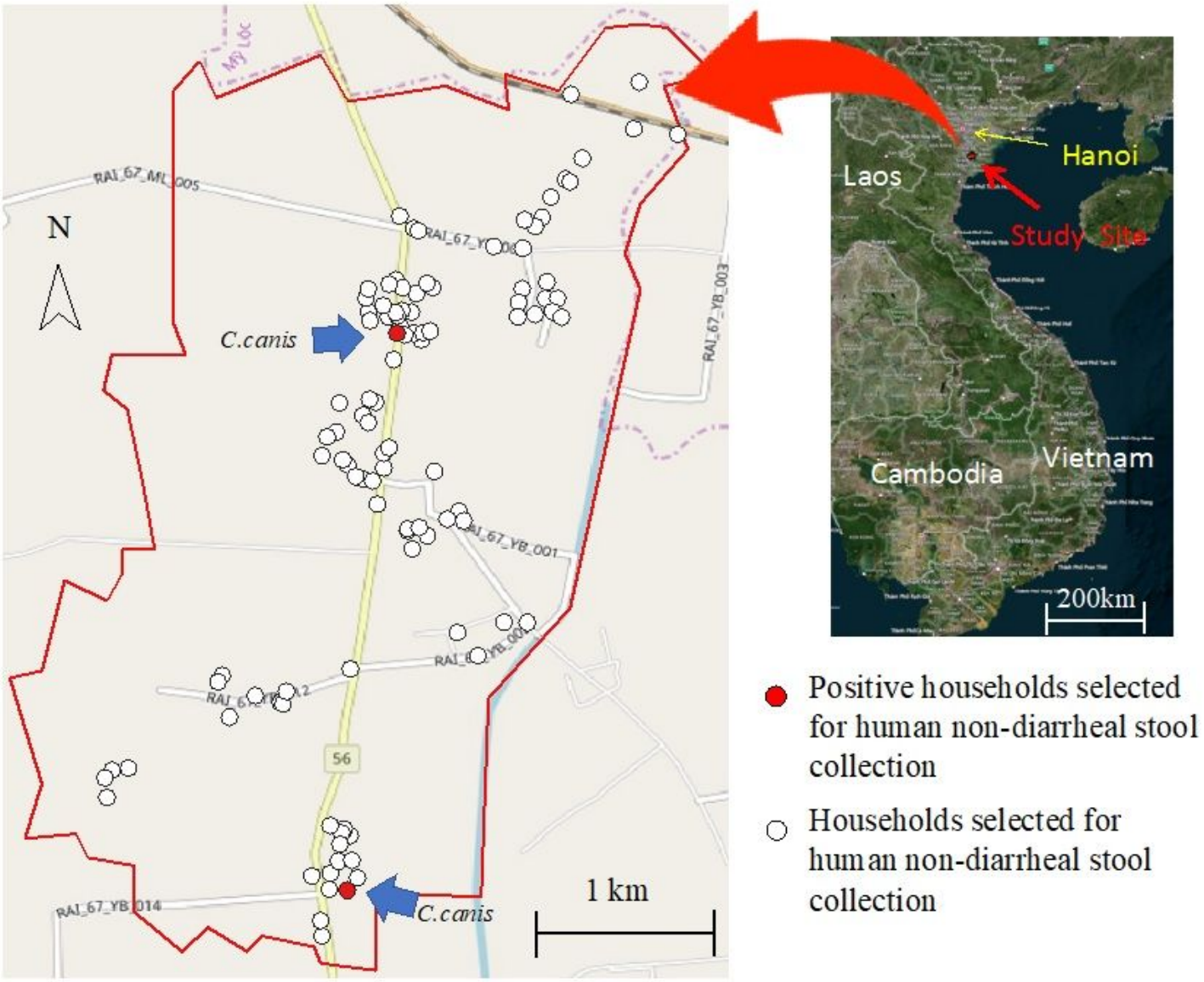


Figure 2

Collection site for human non-diarrheal stool samples. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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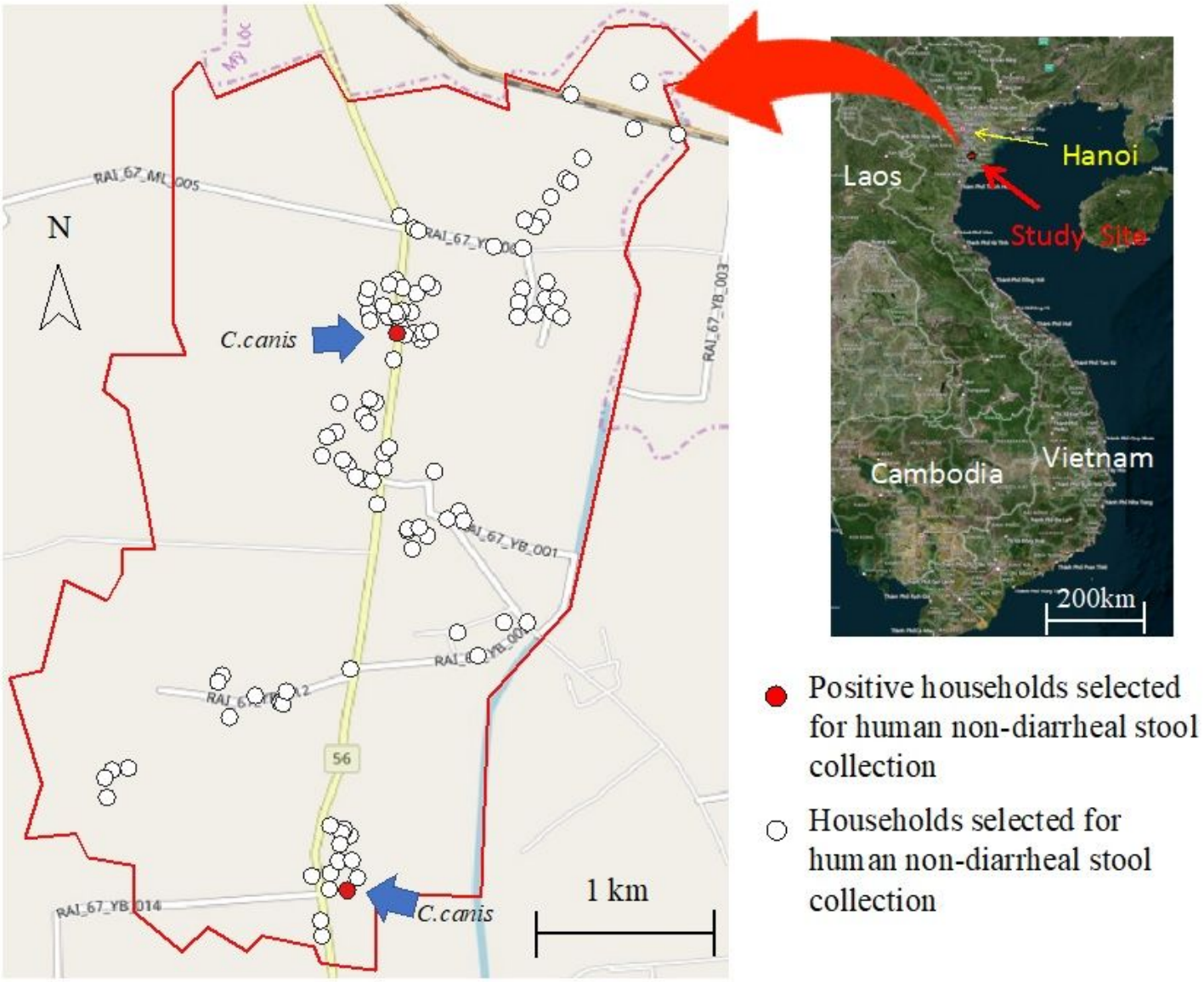

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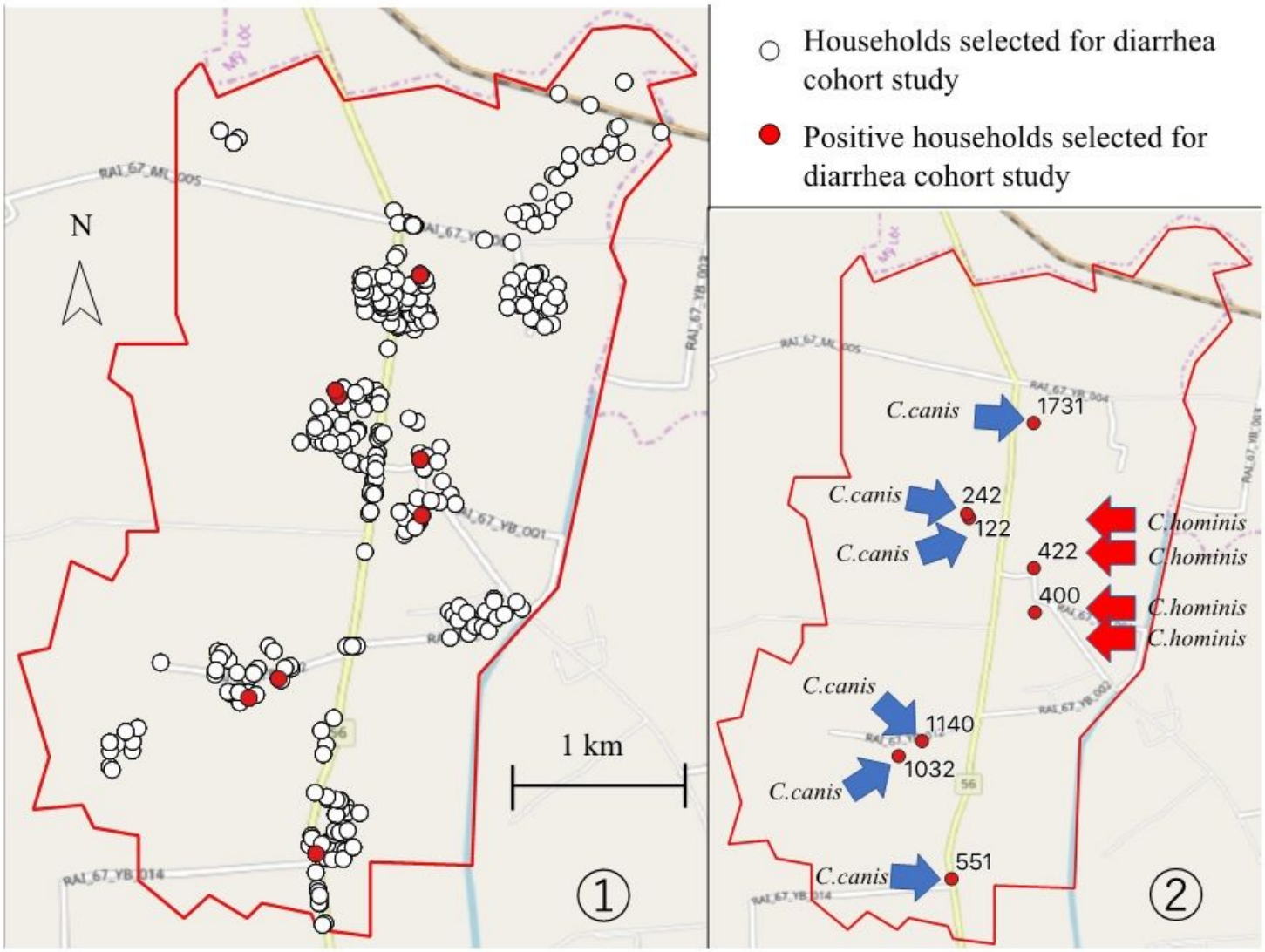
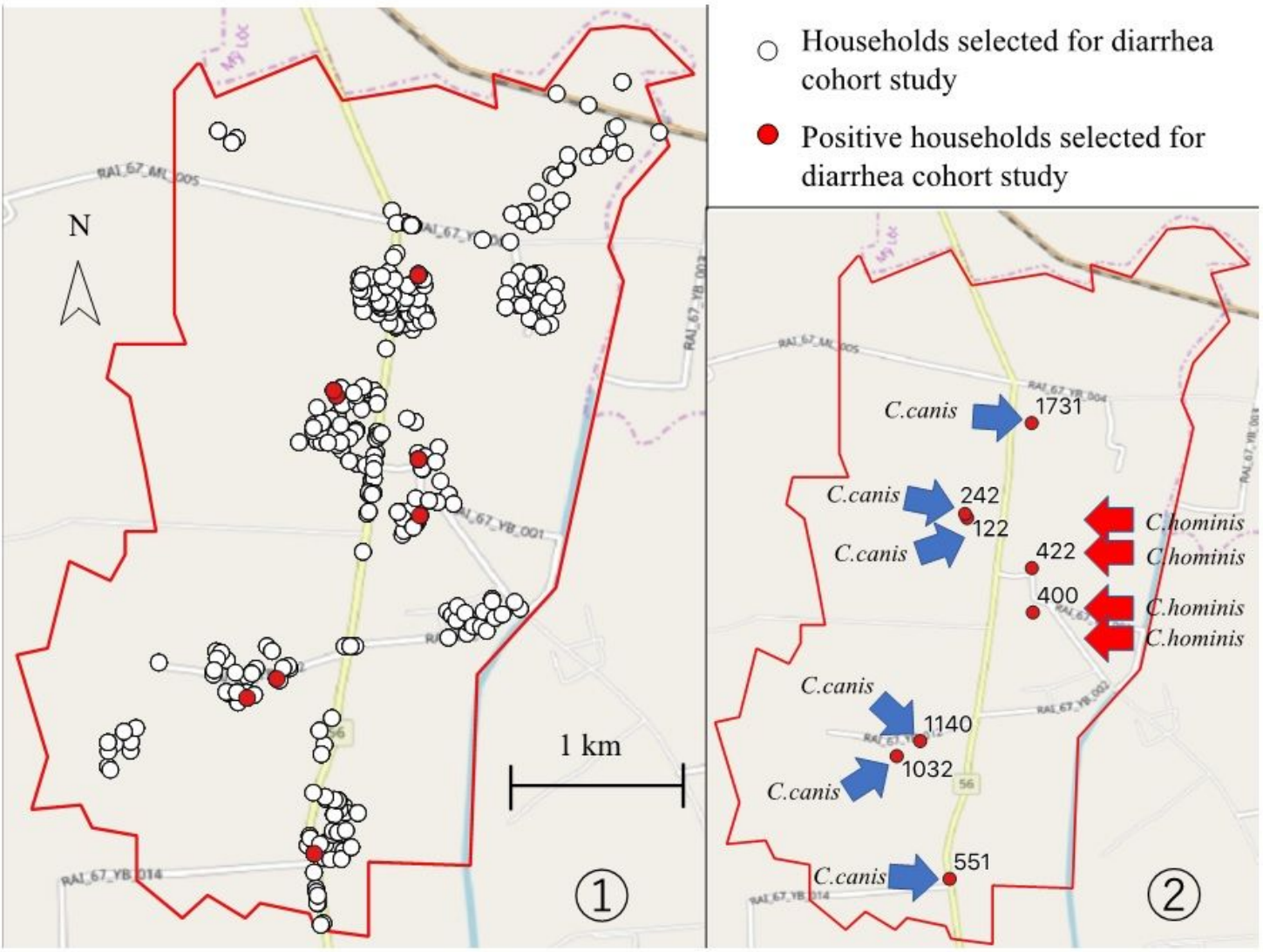


Figure 3

Collection site for human diarrheal stool samples. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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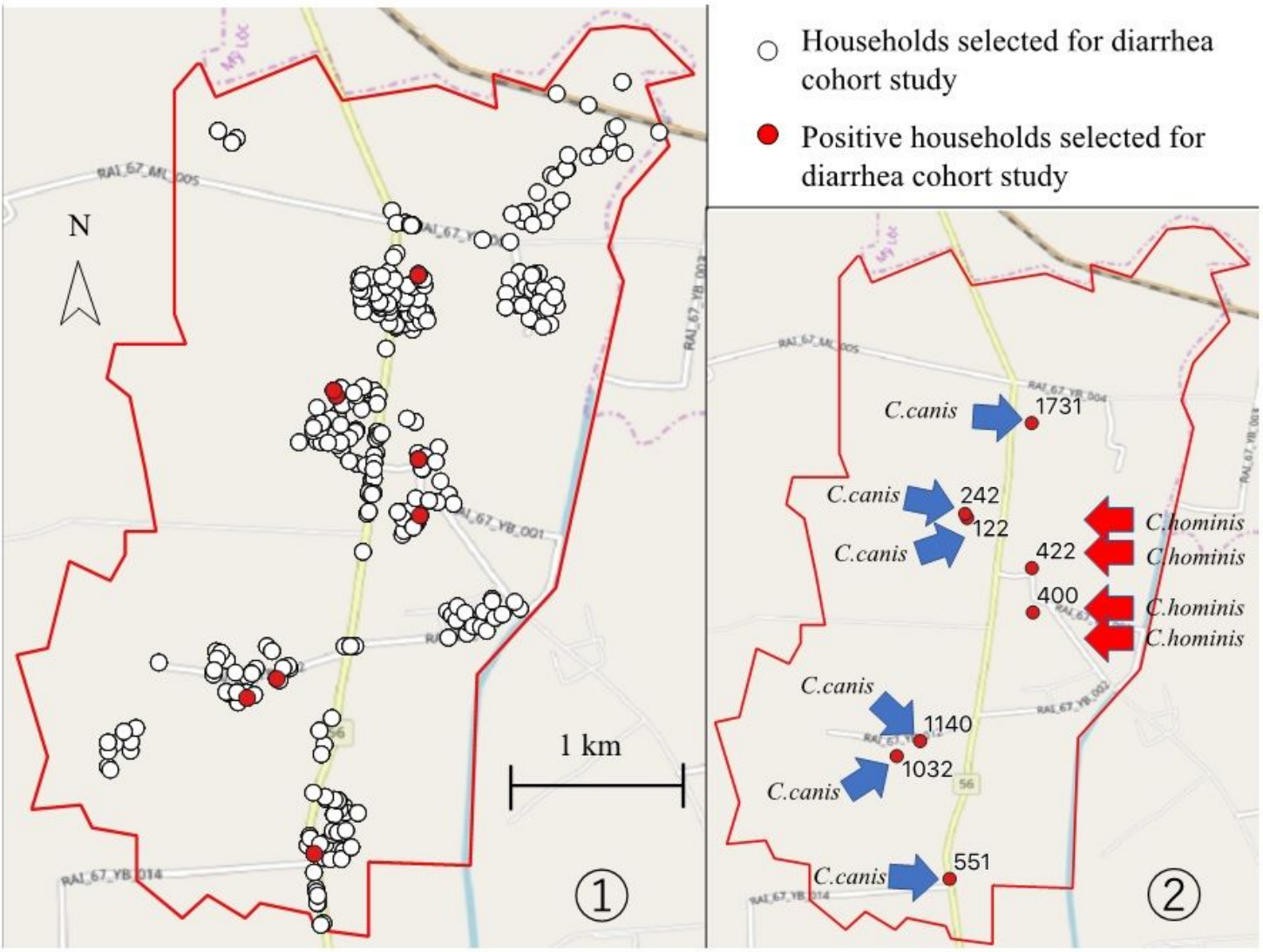

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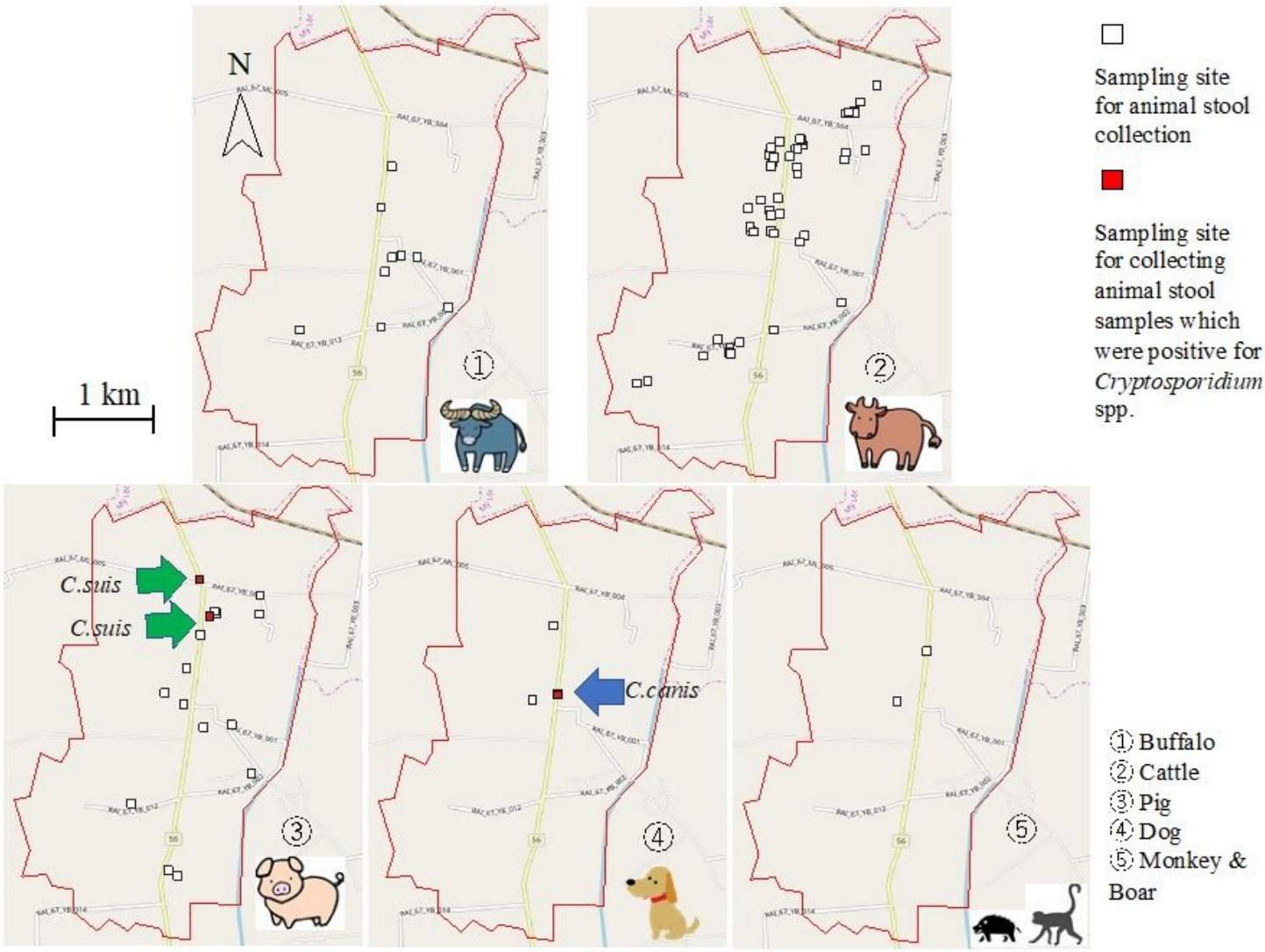
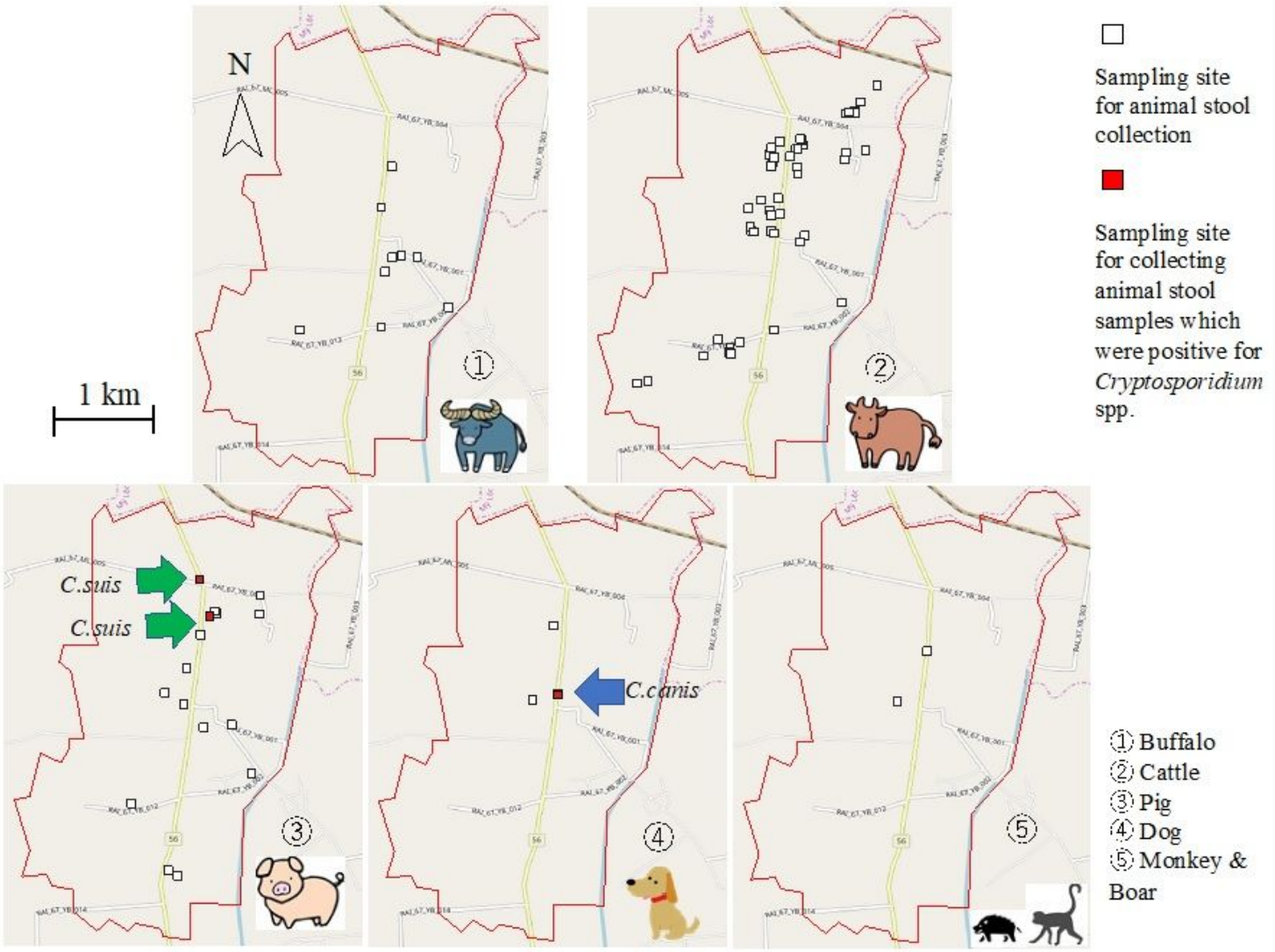


Figure 4

Collection site for animal stool samples. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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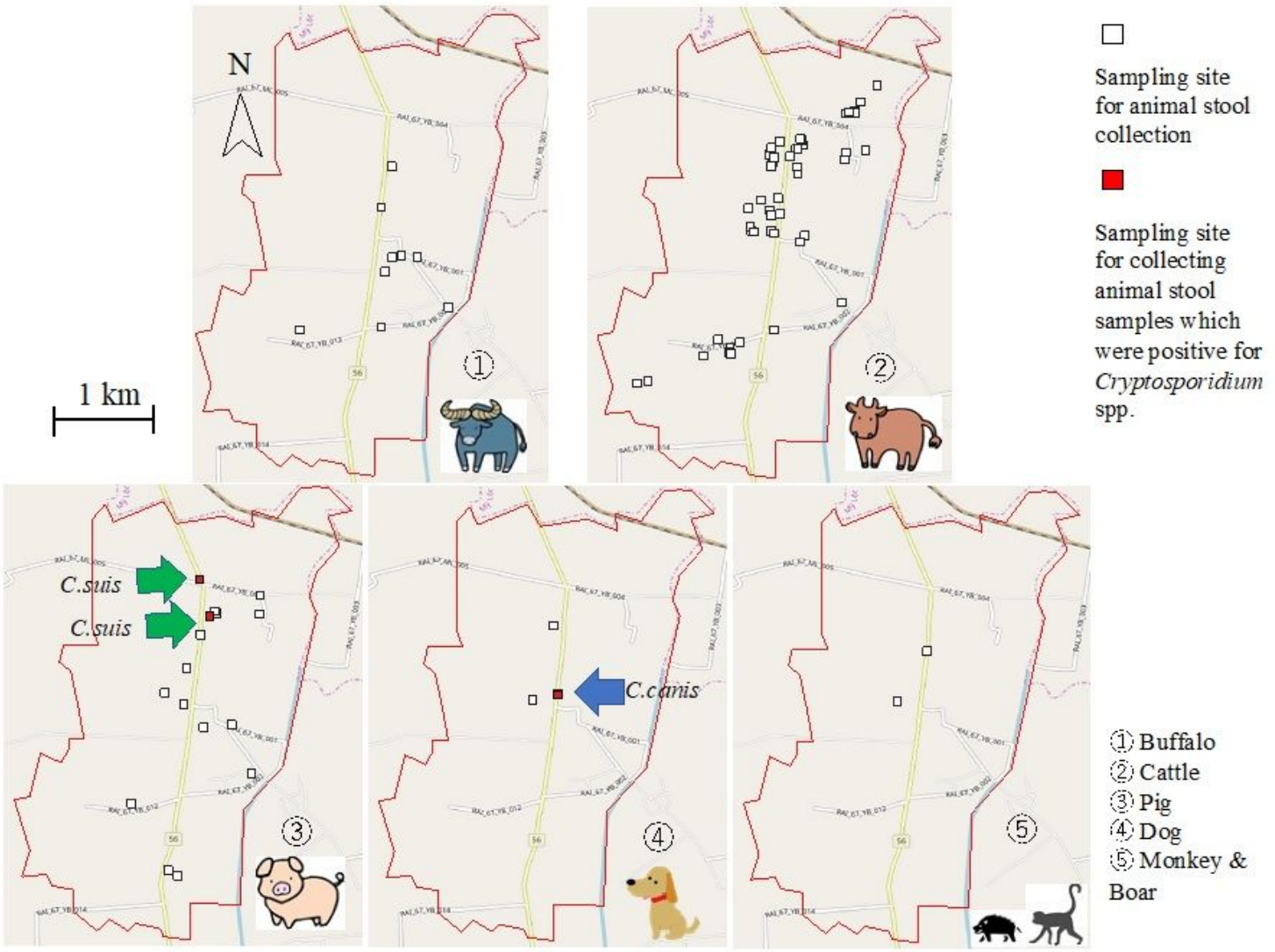

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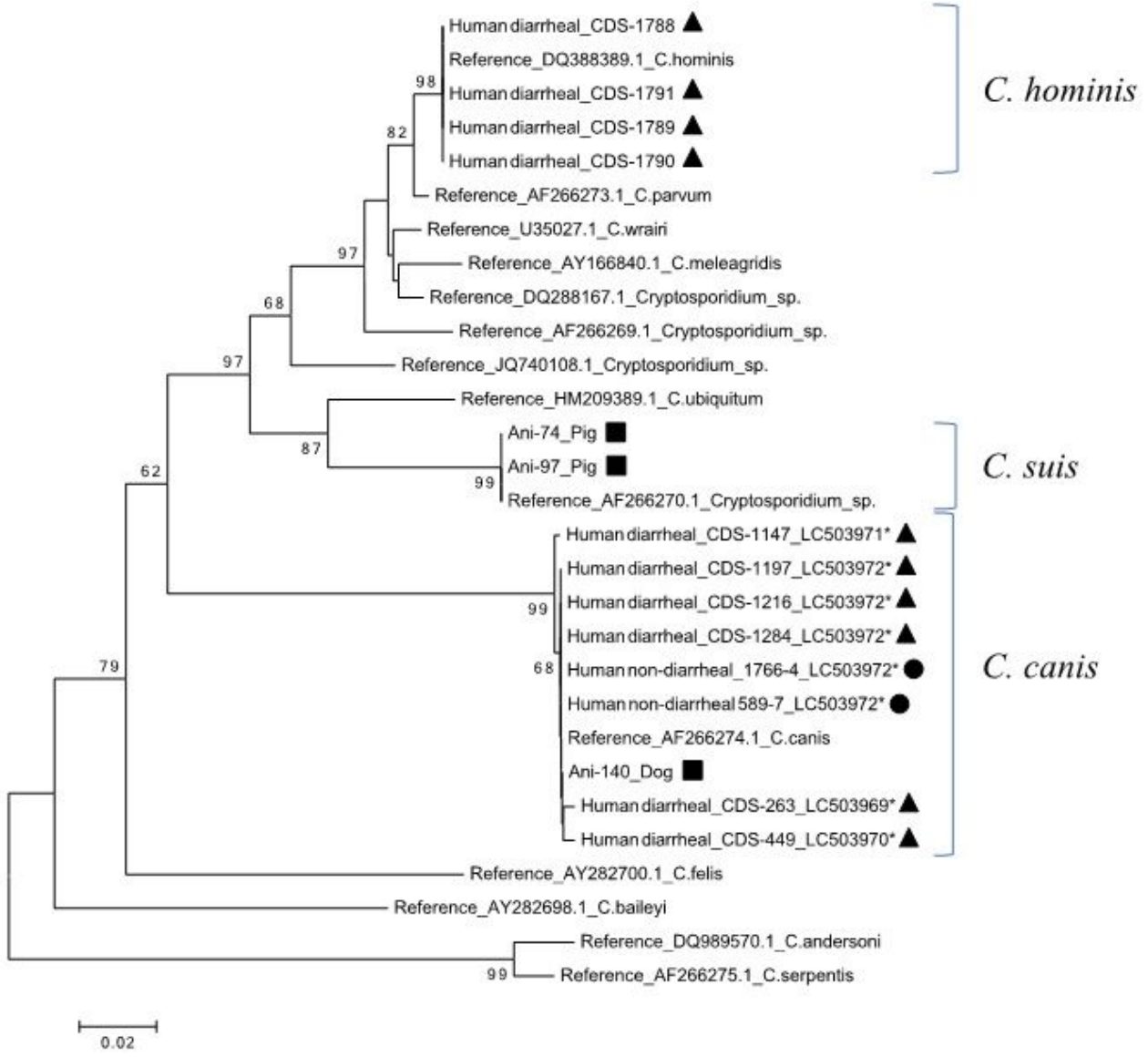
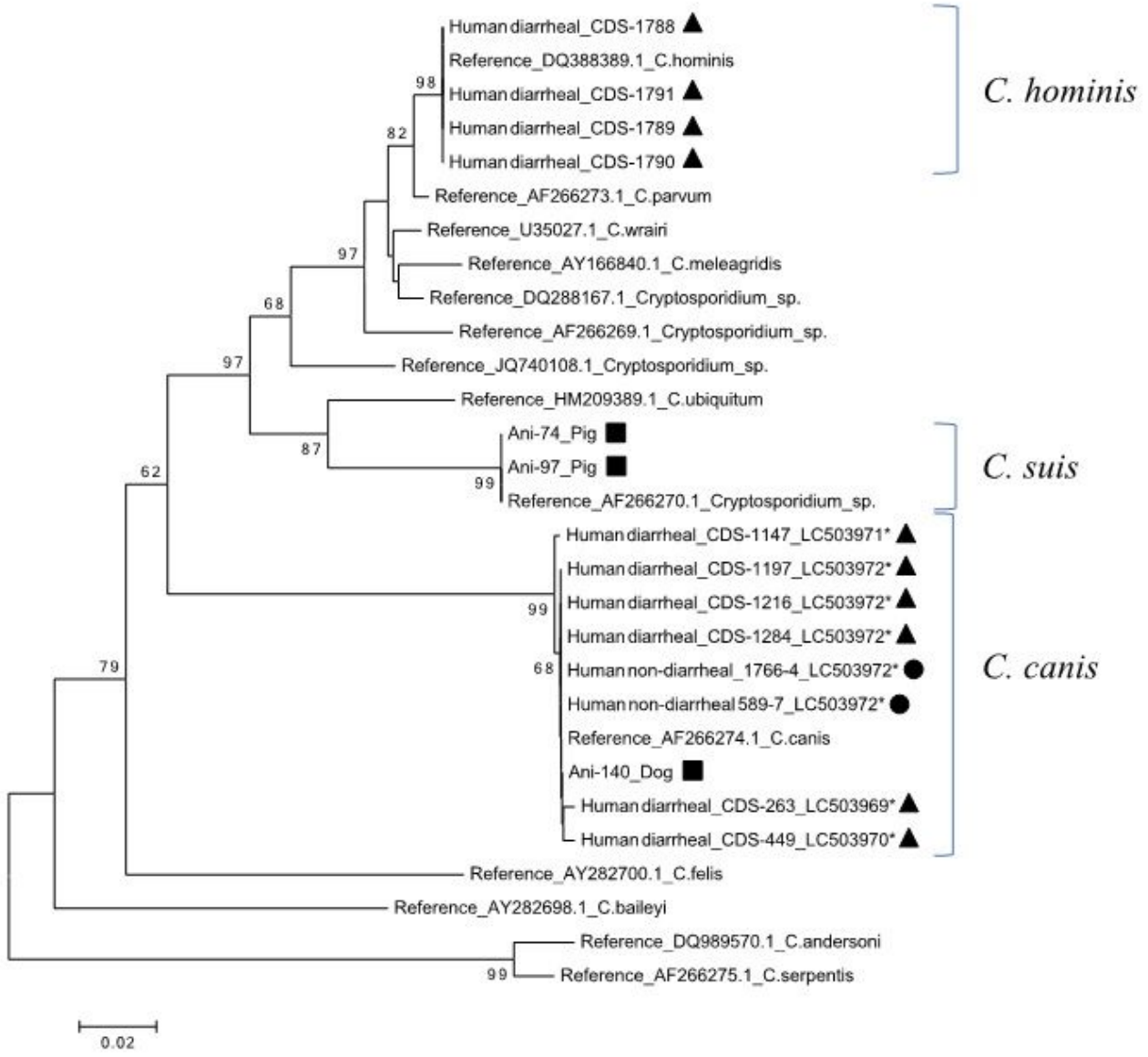


Figure 5

Phylogenetic analysis of partial COWP sequences. Filled squares, circle, and triangles represent animal, human diarrheal, and human non-diarrheal samples, respectively, from this study. Symbol (*) indicates novel sequences newly submitted to GenBank.



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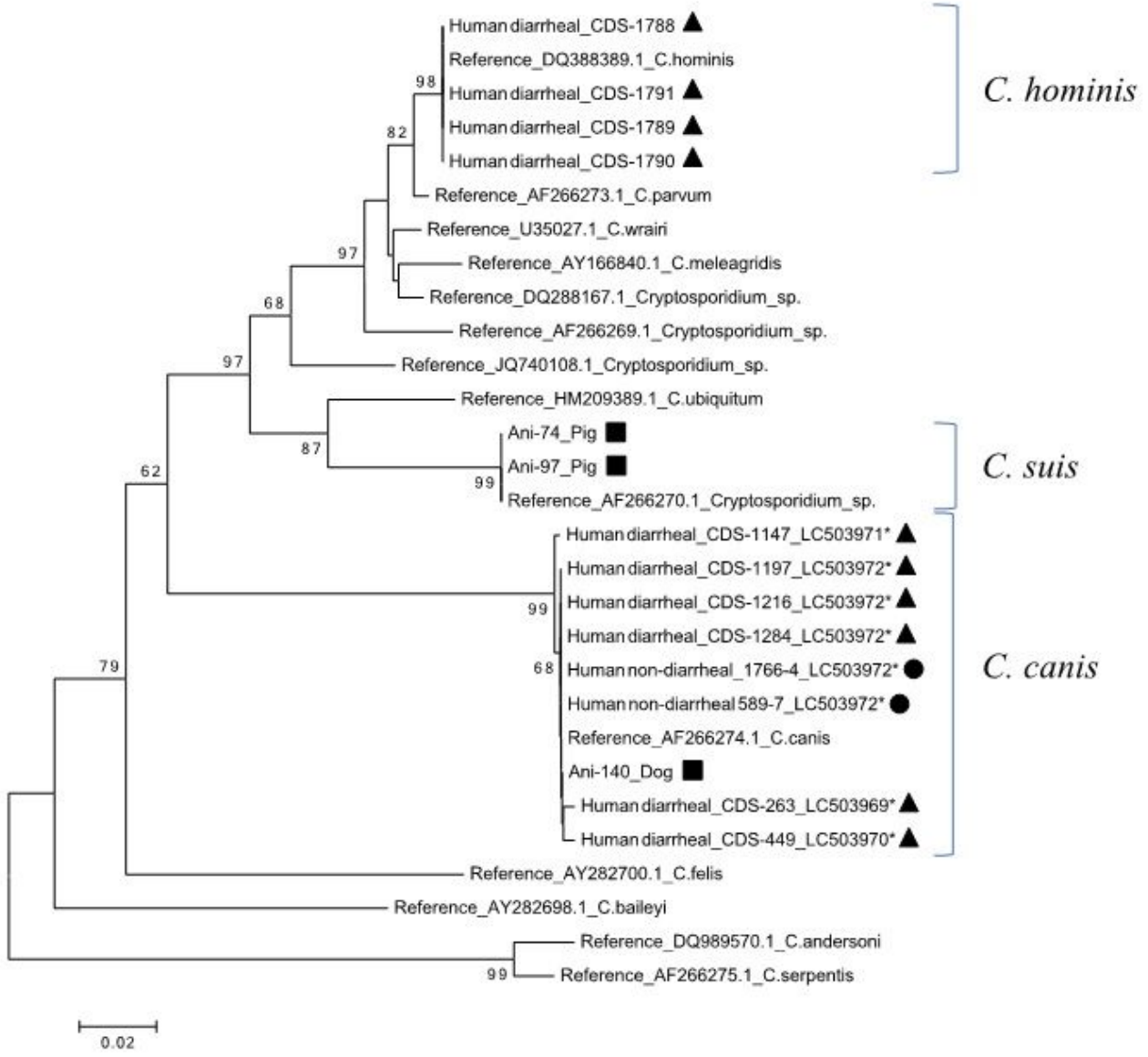

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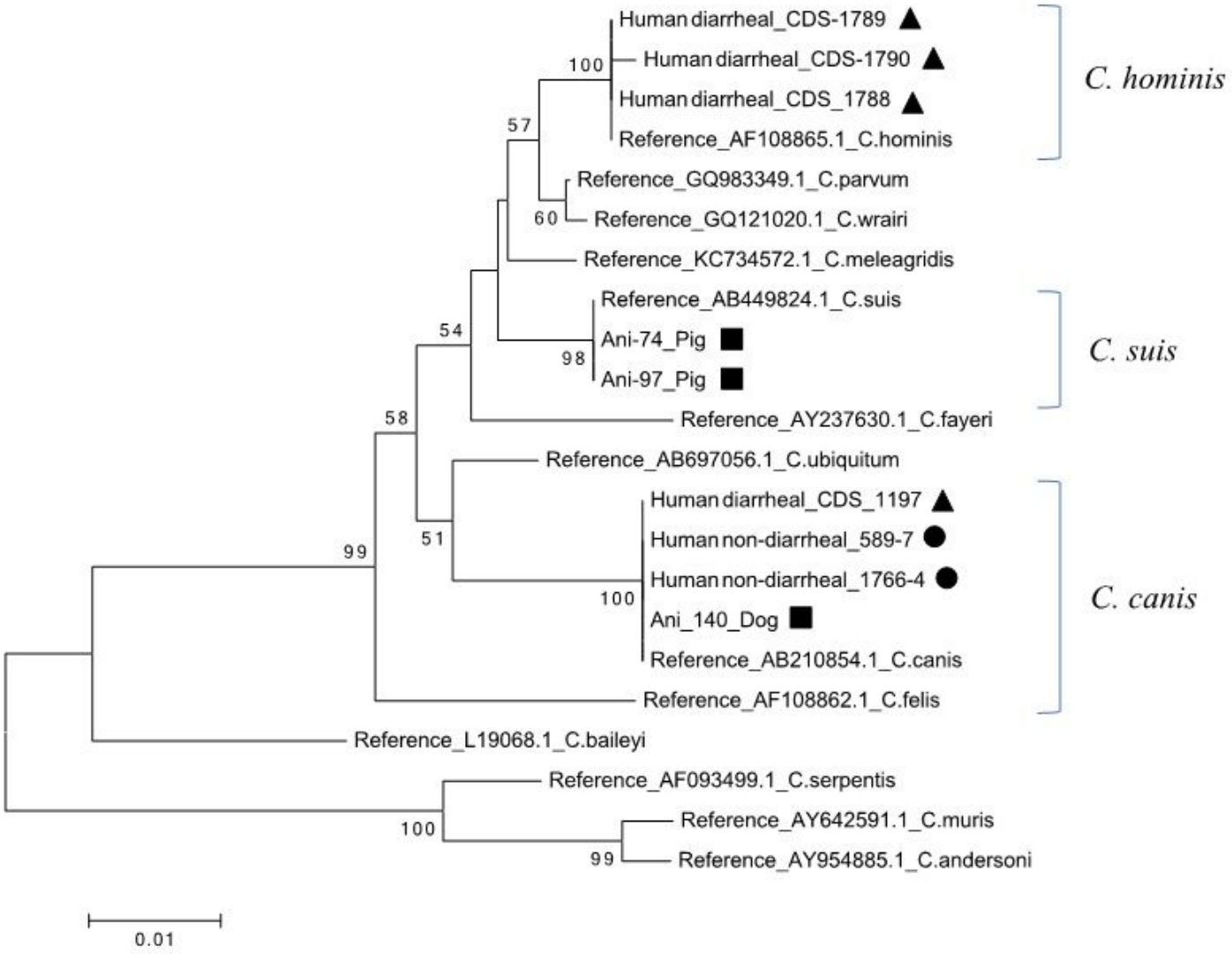

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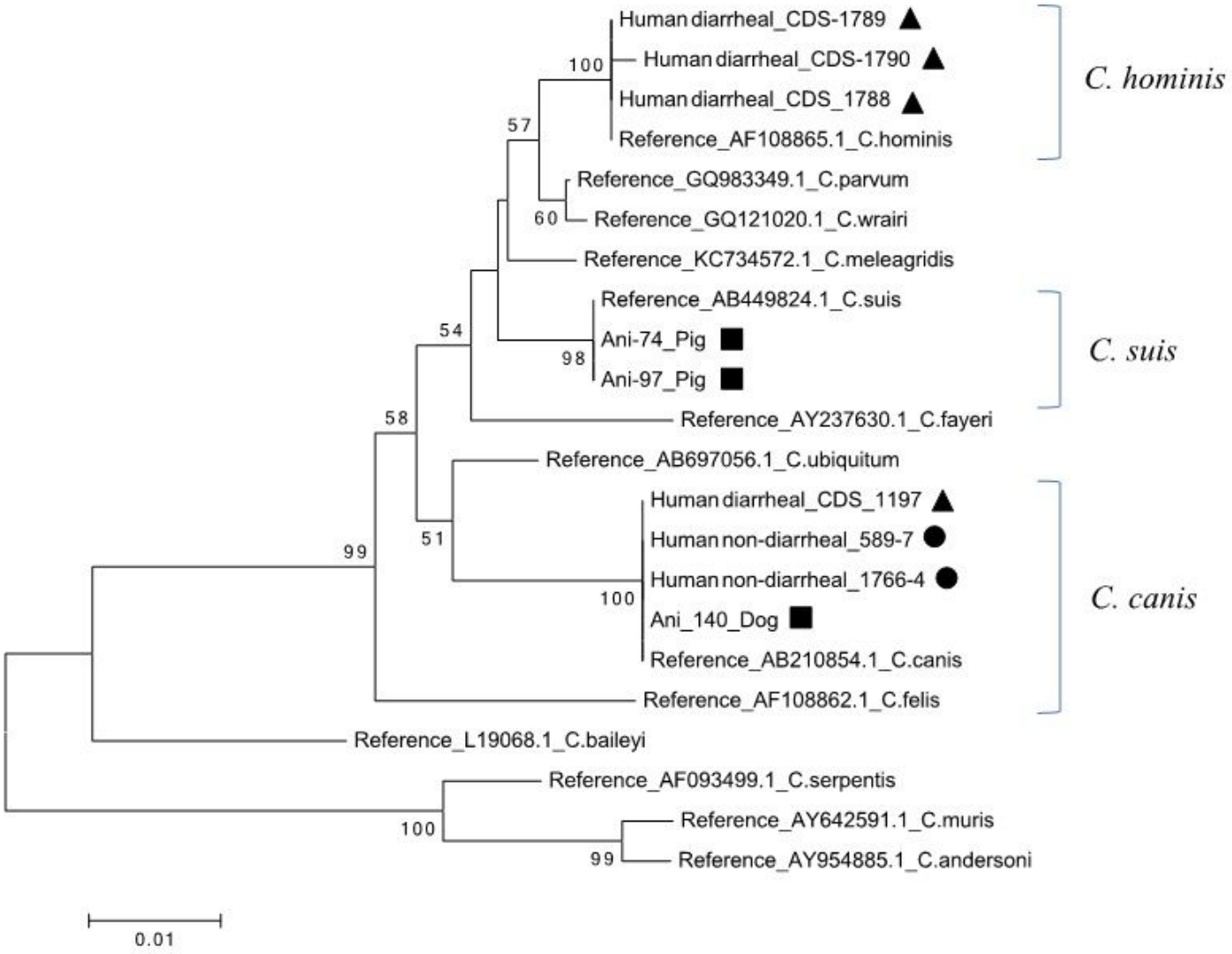
Phylogenetic analysis of partial SSUrRNA sequences. Filled squares, circle, and triangles represent animal, human diarrheal, and human non-diarrheal samples, respectively, from this study. Symbol (*) indicates novel sequences newly submitted to GenBank.



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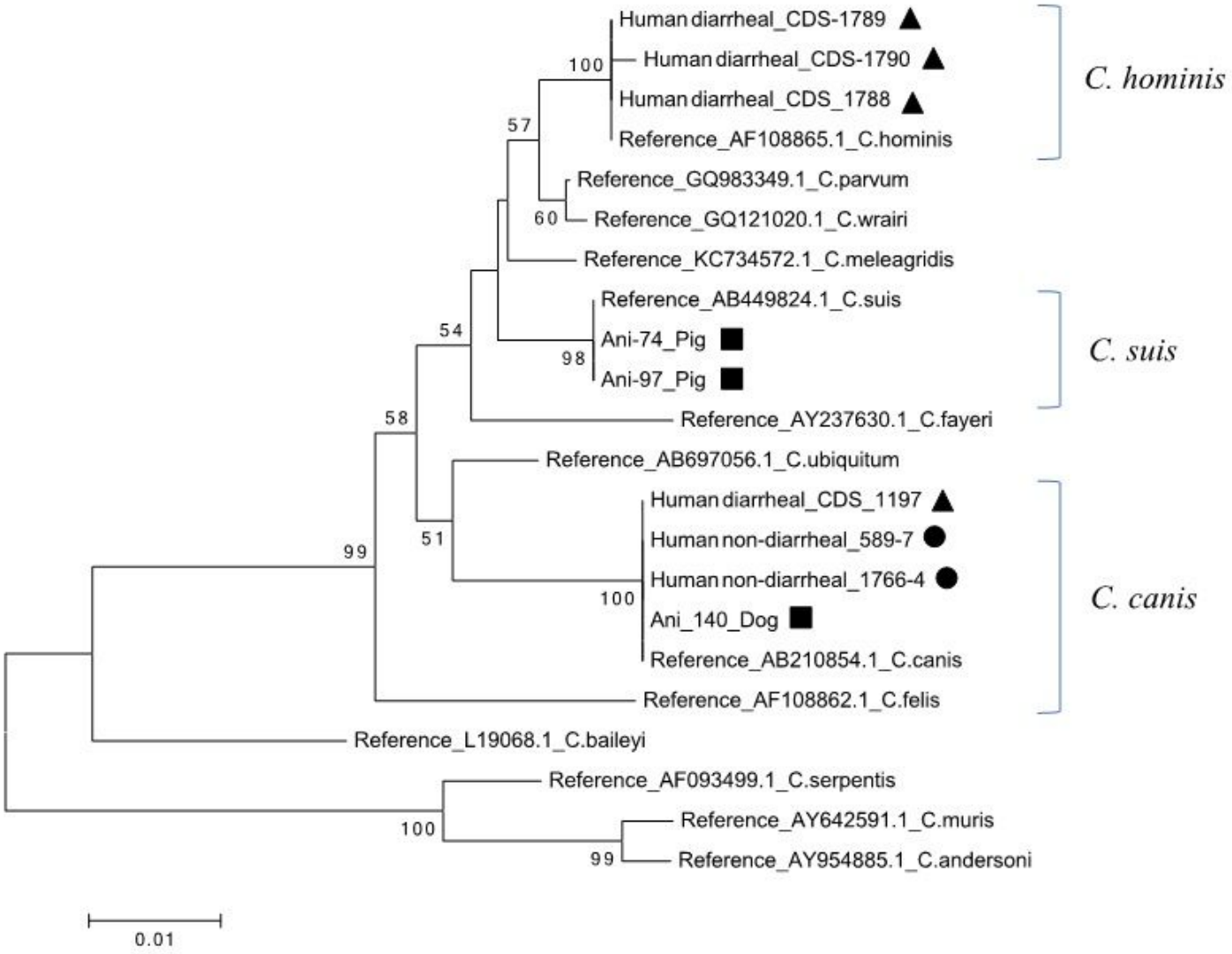

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