ORIGINAL ARTICLE

POTENTIAL CROSS-CONTAMINATION OF SIMILAR *Giardia duodenalis* ASSEMBLAGE IN CHILDREN AND PET DOGS IN SOUTHERN BRAZIL, AS DETERMINED BY PCR-RFLP

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SUMMARY

Giardia duodenalis is an enteric parasite that has distinct genetic groups. Human infections are mainly caused by assemblages A and B, although sporadic infections by assemblages C and D have also been reported. Animals can be infected by a wide range of assemblages (A to H). The aim of this study is to identify the assemblages and sub-assemblages of *G. duodenalis* with zoonotic features in fecal samples of school-aged children, and in dogs that coexist in the same households in *Lages, Santa Catarina*, Brazil. Fecal samples of 91 children and 108 dogs were obtained and *G. duodenalis* cysts were detected in samples from 11 (12.08%) children and 10 (9.25%) dogs. DNA extracted from the 21 positive samples was analyzed by PCR-RFLP, using the *gdh* gene. Results showed the presence of sub-assemblages AI (2/11), AII (4/11), BIII (2/11), and BIV(3/11) among children and AI (5/10) and BIV(3/10) in dogs, with zoonotic characteristics, and the carnivore specific assemblage C (2/10). *G. duodenalis* was found to infect both children and dogs living in the same household, with the same sub-assemblage (BIV) indicating that pet dogs are a potential risk of transmission of *G. duodenalis* to humans.

KEYWORDS: *Giardia duodenalis*; Pet dogs; Zoonotic genotypes; PCR-RFLP.

INTRODUCTION

Giardiasis is a common parasitic disease in humans, with a high worldwide morbidity. In 2004, it was included in the WHO 'Neglected Diseases Initiative', which consists of heterogeneous groups of diseases that impair development and socio-economic improvements[1]. It is present in 2- 5% of the population in industrialized countries and 20-30% in developing countries, with 200 million symptomatic cases and a yearly average of 500 thousand new cases[2,3]. In developed countries, giardiasis is referred to as a reemerging parasitosis due to an increment of diarrhea-related diseases in daycare centers, and epidemics caused by the intake of contaminated water[4]. The presence of *G. duodenalis* in humans is registered mainly in schools, daycare centers and places with large gatherings of individuals.

In children, the disease is spread by ingesting contaminated feces, usually through a hand to mouth contact. Feces of infected children are filled with *Giardia* cysts which are infectious. The cysts are also present on toys, changing tables, and utensils. Many children are not very sick, but can suffer from nutritional deficiencies and weight loss[5,6] and may spread the disease unwittingly. Giardiasis spreads quickly in children care centers and institution for handicapped people.

In dogs, the prevalence varies according to age groups, diagnostic methods, animal life conditions (domestic, stray or sheltered), situation of super populations and if animals are immunocompromised[7]. The coexistence of children and dogs under situations in which basic sanitation is lacking determines the higher prevalence of *G. duodenalis*, and zoonotic contamination, a possibility that cannot be ignored[8].

In the state of *Santa Catarina* (Brazil) studies were conducted showing differences in the prevalence of *G. duodenalis* cysts in fecal samples from school-aged children. Schnack *et al.* (2003)[9] diagnosed *G. duodenalis* cysts (4.3%) in *Criciúma*; Andrade *et al.* (2008)[10] described a prevalence of 18% in children in *Blumenau*; Kunz *et al*., (2008)[11] described a prevalence of 13.8% in children from a municipal school in *Florianópolis*. In *Concórdia* city, prevalences were 15.9% (111/699), 15.2% (58/383) and 8.3% (5/60) in school-aged children in the years of 2000, 2001 and 2002, respectively[12].

In *Lages*, the city in which this study was carried out, the prevalence was 14% (28/200) in children from six Municipal Centers for Children's Education (CEIMs) aged two to six years old[13] and 27% (27/100) in children aged one to 12 years old[14]. In children receiving treatment from the Family Strategic Health Program (ESF), in six suburban

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neighborhoods, *G. duodenali*s cysts were diagnosed in 20% (21/105) of fecal samples[3].

Human samples were found to be infected with the A and B assemblages[15,16] and wild and domestic carnivores presented assemblages A, B, C and D[2], while bovines, caprines, sheep and pigs are infected with the assemblage E[17]. Assemblage F was isolated in felines, assemblage G in rats[16] and assemblage H in marine vertebrates[18].

In the current study, we explored the conditions that have determined the prevalence of zoonotic features of *G. duodenalis* under the same households in *Lages*, *Santa Catarina*, Brazil. This is the first study of *Giardia* genotyping performed in the state of *Santa Catarina* and one of a few conducted in Southern Brazil.

MATERIAL AND METHODS

Fecal sample collection and processing

The research was carried out from July 2010 to December 2011, and recruited 91 children and their pets. Children attended the first to the fifth grades of the Elementary Classes of Municipal Basic Education Schools (EMEB) in the city of *Lages*, from a universe of over 300 students. The neighborhoods of the participant schools were *São Miguel, Santa Helena, Santa Catarina, Sagrado Coração de Jesus, Guarujá, Caroba, Habitação, Tributo* and *Centenário* (Fig. 1).

The research project, number 005-09, was approved by the Committee of Ethics and Research (CEP) in Humans of the University of the *Planalto Catarinense (UNIPLAC).* Parents signed the informed consent after attending a presentation of the research. Weekly visits for detailed explanation of the project were performed, and an epidemiological questionnaire was applied to parents with questions on the social and domestic characteristics of the population (number of children in the household; existence of sewage system and treated water; number of dogs; dogs living inside or outside the house, and if they received any veterinary care). During the visits, identified collecting containers were handed out and dates were agreed for the gathering of the fecal samples. Adults responsible for the children were instructed to collect the samples and to keep them refrigerated.

Samples were kept at 4 ºC with no use of preservatives, for a maximum interval of 48 hours until parasitology exams were performed at the Laboratory of Parasitology at the University of the *Planalto Catarinense (UNIPLAC).* All samples were prepared for microscopic examination after centrifugation and concentration, by a flotation technique with saturated zinc sulfate and a sedimentation procedure. A single fecal sample was obtained from each dog and child, as further sampling was impaired by lack of continued collaboration of the community.

Molecular characterization

G. duodenalis isolates were kept in ethanol 96˚GL, as instructed[19,20]. Samples containing *G. duodenalis* cysts were submitted to DNA extraction, using the ultrasound[21] and glass beads[22,23] techniques and submitted to amplification by means of a semi-nested-PCR using the *gdh* gene and subsequent identification of the assemblages by Restriction Fragment Length Polymorphism (RFLP)[24].The amplification product was 432 bp and the primers External - GDHiF: 5'-CAACGTYAAYCGYGGYTTCCGT-3'; Internal -
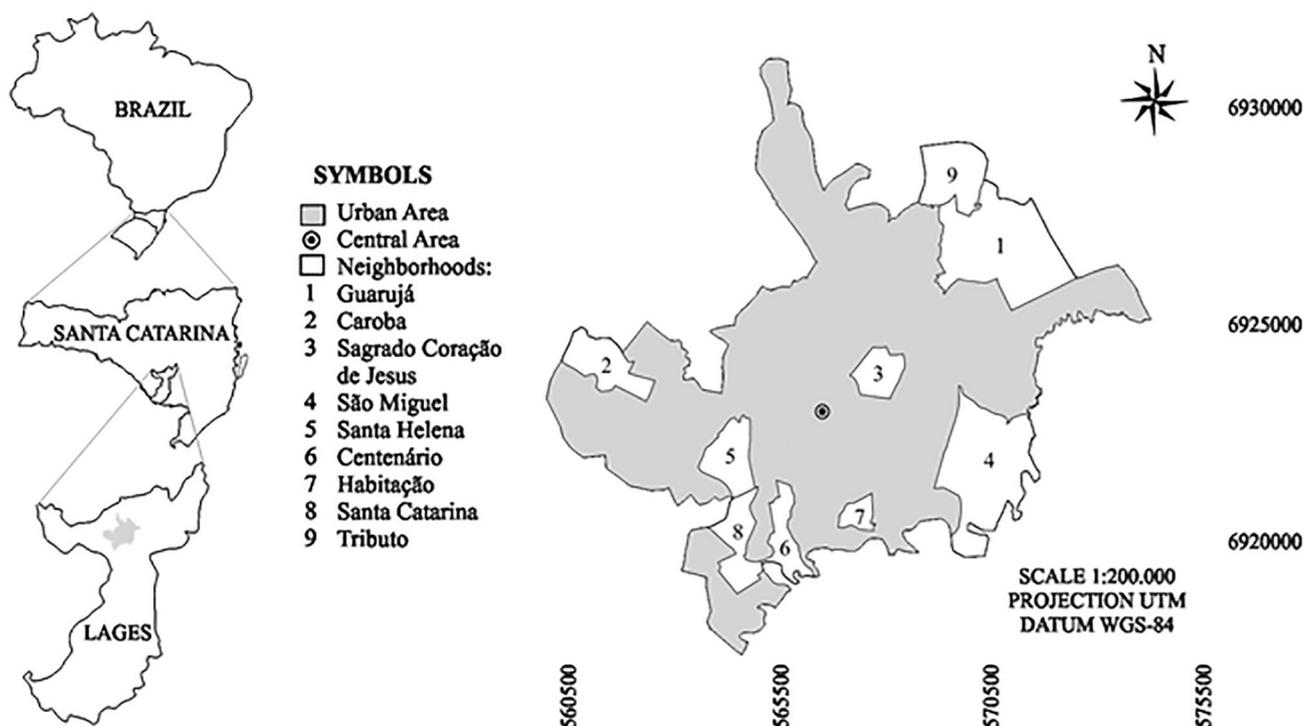

Fig. 1 - Localization of neighborhoods in *Lages, Santa Catarina* in which the samples were colleted for this study.

GDHiF: 5'-CAGTACAACTCYGCTGG-3' and Reverse -GDHiR: 5'-GTTRTCCTTGCACATCTCC-3'. Amplifications were performed in a final volume of 25 µL containing 12.5 pmol of each primer, 300 µM of dNTP, 1.5 mM of $MgCl_2$, 2.5 units of *Tth* DNA polymerase (Promega, Madison, USA), and *Tth* buffer, using the primers GHDiR and GDHe for the first reaction from 3 µL of genomic DNA. In the second reaction (semi-nested PCR), 3 µL of the first reactions were used as template DNA along with 12.5 pmol of the primers GHDiR and GDHiF. The Biocycler thermal cycler (Bioer Technology, Hangzhou, China) was used under the following amplification conditions: 1 cycle at 94 °C for 2 min, 56 °C for 1 min and 72 °C for 2 min. In the second round of amplification, other 55 cycles were performed at 94 °C for 30 sec, 56 °C for 20 sec, and 72 °C for 45 sec, ending with a final extension of 72 °C for 7 min. Amplification products were kept at -20 °C until the genotyping.

Genotyping by RFLP

Amplification products were divided into two aliquots in microtubes, each one containing 20 µL of final volume. In the first microtube, two units of the *Nla* IV enzyme (New England Biolabs, Ipswich, USA) were added, 2.4 µL of the corresponding buffer and 1.2 µL of bovine serum albumin. The second microtube contained two units of the *Rsa* I enzyme (New England Biolabs, Ipswich, USA) and 2 µL of corresponding buffer. Digestion took place at 37°C for 12 hours. Later, DNA was precipitated with absolute ethanol. After RFLP, digested DNA samples were visualized in 2% agarose gels stained with ethidium bromide.

Information on the isolates and the identified genotypes (Table 1) followed the descriptions of Read *et al.* (2004)[24].

Statistical analysis

The χ^2 test of R[25] software (The R Foundation for Statistical Computing, Vienna, Austria version: 25)was used, and the FREQ procedure of the SAS statistical package (SAS Institute Inc, Cary NC-USA- Version: SAS University Edition) using $p \leq 0.05$ significance level and trust interval of 95 % (IC).

RESULTS

Among the 91 fecal samples obtained from children, 52.75% (48/91) were from girls and 47.25% (43/91) from boys; 24.18% of the children were 5- 6 years old; 36.26% were 7- 8 years old; 34.07% were 9-10 years old; and 5.49% were older than 10 years (Table 2). These samples were collected from children in 76 households and showed a positivity of 12.08% for *G. duodenalis,* totaling 11 samples, in the flotation and sedimentation methods. There was no significant difference between boys and girls regarding *G. duodenalis* infection.

From the total of 108 fecal samples obtained from pet dogs, 54.63% (59/108) were from male dogs and 45.37% (49/108) from female dogs. These samples showed positivity of 9.25% for *G. duodenalis,* totaling 10 samples, in the flotation and sedimentation methods. *G. duodenalis*

Table 1

Assemblages and sub-assemblages detected by restriction enzymes in *G. duodenalis* isolates from children and dogs living in the same homes in *Lages*, *Santa Catarina*, Brazil

Assemblages/sub-assemblages	Enzyme (number of restriction sites)	Molecular weight of DNA fragments	Identification of assemblages/ subtypes
AI	*Nla* IV (5)	16, 18, 39, 87, 123, 149	90, 120, 150
AII	*Nla* IV (6)	18, 16, 39, 72, 77, 87, 123	70, 80, 90, 120
BIII	*Nla* IV (2)	18, 123 291	120, 290
BIV	*Nla* IV (2)	18, 123, 291	120, 290
C	*Nla* IV (4)	18, 31, 72, 123, 187	70, 120, 190

Table 2

Parasitological examination of fecal samples from children and dogs infected with *G. duodenalis* living in the same homes in *Lages*, *Santa Catarina*, Brazil. Relationship between sex and age

Age Group (years)	Children		Age (months)	Dogs	
	Female + (n samples)	Male + (n samples)		Female + (n samples)	Male + (n samples)
5 – 6	02 (12)	02 (10)	≤3 – ≤6	01 (06)	02 (05)
7- 8	04 (16)	01 (17)	7 - ≤12	01 (15)	01 (13)
9 -10	01 (16)	01 (15)	> 12	02 (25)	03 (37)
>10	00 (04)	00 (01)	NI*	00 (03)	00 (04)
Total	**07** (48)	**04** (43)	**Total**	**04** (49)	**06** (59)

NI* = Not Informed

was detected in 10.17% of male dogs and in 8.16% of female ones. There was no statistical difference between the sex and age of dogs as far as *G. duodenalis* infection is considered (Table 2).

Regarding both diagnostic methods, 21 samples were positive considering pooled results for children and dogs, and DNA extraction of *G. duodenalis* positive samples was successful in 100% of cases by means of the glass beads method that was used in 28.57% (6/21) of the isolates and by ultrasound in 71.43% (15/21) of the isolates.

The diagnosed assemblages in the samples of children and dogs are shown on Table 3. Results after the 2% agarose gel electrophoresis are depicted in Figure 2. Of the 21 *G. duodenalis* isolates, 52.38% (11/21) were of the assemblage A: 63.64% (7/11) in sub-assemblage AI and 36.36% AII (4/11). The assemblage B was detected in 38.10% (8/21) of the samples: 75% (6/8) belonging to the sub-assemblage BIV and 25% to BIII (2/8). Finally, 9.52% (2/21) were assemblage C. A total of 90.48% (19/21) of the samples presented zoonosis features depicted by assemblages A and B, since assemblage C is specific to carnivores and represents no risk of human infection. None of the children with infected samples had diarrhea.

Regarding the sanitary conditions, all households had treated water and sewage systems, but only 7.9% (6/76) of the animals received veterinarian care. The number of children in households varied only slightly: 1 (19.74%), 2 (40.79%), 3 (31.58%) and 4 (7.90%). No statistical difference was found between the number of children per households relative to the children infected with *G. duodenalis* ($p > 0.05$). The average number of animals per household was 1.42. Risk of infection was observed in animals living both indoors and outdoors, but it was not statistically significant ($p > 0.05$). Concerning veterinary care, only 7.9% of the respondents reported having ever taken their dogs to a veterinarian.

DISCUSSION

Giardia genotype distribution studies have demonstrated, in many countries, the occurrence of diversity in the prevailing *G. duodenalis*
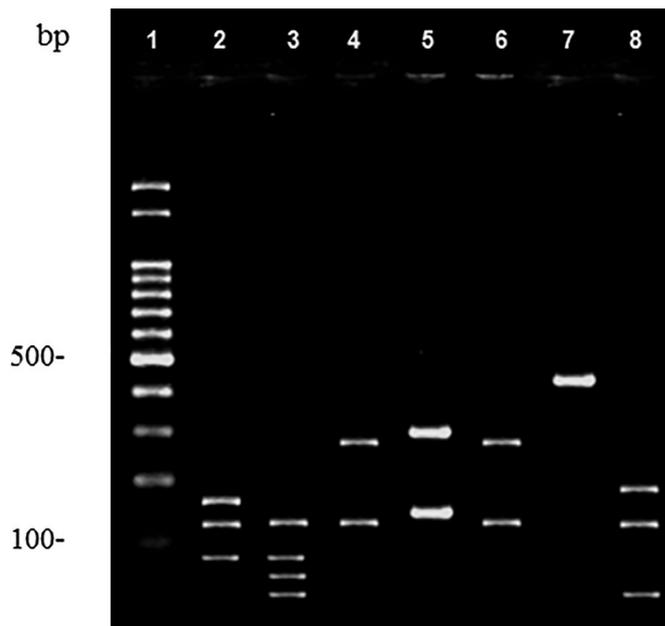

Fig. 2 - Ethidium bromide-stained 2 % agarose gel showing DNA amplification of the *gdh* gene from each of the genetic Assemblages: AI, AII, B, BII, BIV and C, digested with *Nla* IV/*Rsa* I. Lane 1, Molecular weight marker 100 bp; Lane 2, Assemblage AI; Lane 3, Assemblage AII; Lane 4, Assemblage B; lane 5, Assemblage BIII; lane 6, Assemblage B; lane 7, Assemblage BIV; lane 8, Assemblage C.

assemblages in humans and dogs [26,27]. The assemblage and sub-assemblage prevalence vary considerably from country to country, and among different studies, although assemblage B appears to be the most common in epidemics[26,28]. However according to Abe *et al.*, (2005)[29] there is no consensus on this matter. In some areas the assemblage A may be more common, as it aggregates *G. duodenalis* isolates of human and animal sources that may be involved in a zoonotic transmission, while the assemblage B is more related to human infections.

Table 3
Classification of genotypes of *G. duodenalis* isolates in children and dogs living in the same homes in *Lages*, *Santa Catarina*, Brazil

Child	Assemblage	Enzymes	Dog	Assemblage	Enzymes
1	BIV	*Rsa* I; *Nla* IV	1	C	*Nla* IV
2	AI	*Nla* IV	2	BIV	*Rsa* I; *Nla* IV
3	AI	*Nla* IV	3	AI	*Nla* IV
4	AII	*Nla* IV	4	BIV	*Rsa* I; *Nla* IV
5[a]	BIV	*Rsa* I; *Nla* IV	5[a]	BIV	*Rsa* I; *Nla* IV
6	AII	*Nla* IV	6[b]	AI	*Nla* IV
7[b]	BIII	*Rsa* I; *Nla* IV	7[b]	AI	*Nla* IV
8[c]	BIII	*Rsa* I; *Nla* IV	8[b]	AI	*Nla* IV
9	AII	*Nla* IV	9[c]	C	*Nla* IV
10	AII	*Nla* IV	10	A1	*Nla* IV
11	BIV	*Rsa* I; *Nla* IV		-	-

[a,b,c] samples from the same household.

Sprong *et al.* (2009)[30] conducted a study to identify *G. duodenalis* assemblages among humans and animals from various public institutions and hospitals in Europe. In this study they identified the sub-assemblage AI in 73% of fecal samples collected from dogs and 25% of samples from humans; sub-assemblage AII was observed in 27% of dogs and 75% of humans; sub-assemblages BIII and BIV were observed in 27% and 73% of dogs and 56% and 44% of humans, respectively. These results corroborate the ones of our study, as we detected a lower prevalence of sub-assemblage AI among humans (18.18%) when compared to dogs (50%). Sprong *et al.* (2009)[30] also registered the prevalence of *G. duodenalis* assemblages among humans throughout the world: sub-assemblage AI was more frequently observed in Australia (92%), AII in Europe (86%), BIII in Africa (81%) and South America (79%), and BIV in North America (86%). Different prevalence rates were observed in fecal samples of children living in *Lages*, with higher rates of sub-assemblages AII (36.36 %) and BIV (27.27%), but lower rates of the sub-assemblage BIII (18.18%) as previously described in South American populations. This difference can be explained because in our work the focus was not only on the children, and our sample was more restricted, while in Sprong *et al.* (2009)[30] human populations were more numerous.

Some epidemiological studies have demonstrated the importance of the dog as a natural reservoir of infection for humans[31,32,33] since the dog-man cycle is major route of contamination. There was a predominance of Assemblage A genotypes of G. duodenalis in dogs as well as in humans in our study, corroborating a number of previous reports[27,34]. In this study the sub-assemblage AI was found in both children and dogs, although not simultaneously at the same household.

The fact that the sub-assemblage BIV was identified in a single child and a dog sharing the same household is also indicative of direct (human-to-dog) or indirect (e.g. through a contaminated drinking water source) transmission. In this case the dog lived outside of the domestic environment. The hypothesis that could corroborate the protozoan transmission cycle would be through water, but the water distributed to all sampled residences was considered of excellent quality, distributed by the city water system, and the river where water is collected is considered class I according to CONAMA (National Environment Council). Thus, the dog may have been infected by ingesting untreated water in the neighborhoods since it had access to the streets.

In this study we showed that assemblage A had a higher prevalence than the assemblage B in isolated samples from children and dogs, which correlates other Brazilian studies. Souza *et al.* (2007)[35] diagnosed sub-assemblages AII (74.4%) and assemblage B (21.6%) in 37 and 27 samples from human and dogs, respectively, in various municipalities in the state of *São Paulo*, Brazil. Volotão *et al.* (2007)[3] analyzed 366 fecal samples from children (n = 310) and adults (n = 56) and 28 from dogs and cats in *Rio de Janeiro* and detected the presence of AI and AII sub-assemblages in fecal samples.

Sub-assemblage AII was not found in dogs and was the most diagnosed in infected children in our work. Khan *et al.* (2011)[36] showed differences between sub-assemblage AI and AII relative to their hosts, so that animals are mostly infected by AI while humans by AII. *G. duodenalis* isolates differ in pathogenicity and other biological features, yet few studies have evaluated these relationships[37]. For Read *et al.* (2002)[26] assemblages A and B seem to be more diagnosed in epidemic situations; they also mentioned an apparent correlation of *G. duodenalis* assemblage A to intermittent diarrhea, and assemblage B to persistent diarrhea, suggesting that the assemblage A would prevail in asymptomatic patients. Following these observations, the molecular results obtained in *Lages* showed that 52.38% of participants were diagnosed with the assemblage A, however, with no diarrhea. For Mohammed Mahdy *et al.* (2009)[38] this clinical presentation does not obey the expected behavior since the assemblage B caused symptoms of giardiasis in a Malaysian aboriginal tribe. Whether molecular and epidemiological data are related to clinical illness (presence or absence of symptoms) is not totally clear and requires further studies. Ayidin *et al.* (2004)[39] observed that in patients in Turkey, diarrhea was associated with assemblage A isolates, and B with asymptomatic patients, while Minvielle *et al.* (2008)[40], in Argentina, observed that patients infected with the *G. duodenalis* AII sub-assemblage presented few symptoms of giardiasis, and patients with the B assemblage were either asymptomatic or polysymptomatic. Sahagún *et al.* (2008)[41], in Spain, observed that AII assemblage isolates were diagnosed in patients with diarrhea, and those with B assemblage were asymptomatic.

As far as the clinical manifestations and *G. duodenalis* assemblages are considered, there is no consensus. Haque *et al.* (2009)[42] mentioned that the B assemblage may be associated with milder or self-limiting diarrhea, yet the presence of other enteric parasites may influence the presence of diarrhea.

According to Prado *et al.* (2003)[43], the diversity of intestinal parasites among scholars is an indication of lack of information about the mechanism of transmission of parasites. In this study, there was a decrease in the incidence of *G. duodenalis* among school-aged children when compared to prior studies conducted in *Lages* by Arruda *et al.*, (2008)[14] (27%) and Almeida *et al.*, (2010)[44] (20%), albeit these studies had been conducted in only one of the city's neighborhoods and at municipal schools, respectively.

Assemblages with zoonotic features were detected, suggesting the existence of a transmission cycle between child and dog in the same households. The decrease in *G. duodenalis* prevalence in the studied population of *Lages* may be due to improved life quality, the implement of the Family Health Strategy Program (ESF) in the city neighborhoods, water treatment and basic sanitary measures in most households.

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CONFLICTING INTERESTS

There are no conflicts of interest.

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