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Use of laboratory methodologies to confirm an outbreak of acute diarrheal disease caused by multiple pathogens

Uso de metodologias laboratoriais para confirmação de surto de doença diarreica aguda causada por múltiplos patógenos

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ABSTRACT

The aim of this study was to investigate an outbreak caused by protozoa, which occurred in a municipality in the Brazil southern region. The investigations were carried out analyzing 47 fresh stool samples and 26 water samples by parasitological and molecular methods, as well as, direct immunofluorescence. After the filtrations of water samples and purification of stool samples, the concentrates were evaluated microscopically for presence of parasites. Molecular analyses were performed by polymerase chain reaction (PCR) for DNA detection of *Giardia* spp., *Cryptosporidium parvum*, *C. hominis* and *Cyclospora cayetanensis*. Out of 26 water samples, 30.8% (8/26) had waterborne protozoa and *C. cayetanensis* was the most prevalent (15.5%). Out of the 47 stool samples, 23.4% (11/47) were infected with *C. cayetanensis* and *Giardia* spp. The results showed that backwash water samples from filters of the Water Treatment Station were contaminated with *C. cayetanensis*, *C. hominis* and *Giardia* spp., suggesting the contamination of water sources with human waste brought by sewage. These results show the importance of protozoa investigation in water and stool samples by laboratory methodologies principally in outbreaks causing acute diarrheal disease.

Keywords. Waterborne Diseases, Diarrhea, Protozoan Infections, Giardia, Cryptosporidium, Cyclospora.

RESUMO

O objetivo do presente estudo foi investigar um surto causado por protozoários, ocorrido em um município da região sul do Brasil. As investigações foram realizadas analisando 47 amostras de fezes frescas e 26 amostras de água por métodos parasitológicos, moleculares e de imunofluorscência direta. Após as filtrações das amostras de água e purificação das amostras de fezes, os concentrados foram avaliados microscopicamente a procura de parasitas. A seguir, foram analisadas, pela reação em cadeia da polimerase (PCR), a detecção de DNA de *Giardia* spp., *Cryptosporidium parvum*, *C. hominis* e *Cyclospora cayetanensis*. Das 26 amostras de água, 30,8% (8/26) apresentaram protozoários de veiculação hídrica, sendo que, *C. cayetanensis* foi o mais prevalente (15,5%). Das 47 amostras de fezes, 23,4% (11/47) estavam infectadas por *C. cayetanensis* e *Giardia* spp. Os resultados mostraram que as águas de retrolavagem dos filtros da Estação de Tratamento de Água estavam contaminadas com *C. cayetanensis*, *C. hominis* e *Giardia* spp. sugerindo a contaminação dos mananciais com dejetos humanos trazidos pelo esgoto. Estes resultados mostram a importância da investigação de protozoários em água e fezes por metodologias laboratoriais, principalmente em surtos que causam doença diarreica aguda.

Palavras-chave. Doenças Transmitidas pela Água, Diarreia, Infecções por Protozoários, Giardia, Cryptosporidium, Cyclospora.

INTRODUCTION

Diarrheal infections cause high health impact in low-income populations, especially in developing countries and children under five years old that are the most affected[1]. All diarrheal diseases are classified as acute or persistent. However, those caused by bacteria, viruses and parasites cause severe diarrhea that are produced by sources of environmental, zoonotic or human contamination[2,3]. The occurrence of the majority of diarrheal outbreaks worldwide is caused by transmission of these pathogens by water and food[4-6].

In recent years, different factors have been related to the spread of emerging pathogens. These factors include the increase of pathogens contamination in drinking water and food; the human susceptibility to these pathogens; changes in treatment technology of drinking water; and globalization[4]. The documented outbreaks demonstrate that people of all ages can be infected. However, children, young and elderly people can develop more severe clinical symptoms[5].

The sources of freshwater used by man, such as wells, rivers, streams and lakes suffer a continuous and increasing process of degradation due to the dumping of sewage, animal stools (wild and for production). In addition, the resulting waste from industrial activities cause contamination in public water supply[7,8].

Risk factors such as house place, age, ingestion of raw vegetables and quality of drinking water are associated to diarrhea caused by parasites transmitted via the fecal-oral route as infections caused by *Cryptosporidium* spp., *Giardia* spp. and *Cyclospora cayetanensis*[8-11].

As Instituto Adolfo Lutz (IAL) is the "Laboratório Central de Saúde Pública" in São Paulo (LACEN/SP) normally is requested to confirm the laboratory diagnosis from other Brazilian laboratories. In 2019, the epidemiological surveillance from a municipality located in the southern region of Brazil requested technical/scientific support in order to investigate a wide-ranging acute diarrheal outbreak that persisted for 4 months. The clinical and epidemiological characteristics appointed to infectious causes involving multiple pathogens, including intestinal protozoa, which were transmitted by treated drinking water.

This article presents the results of the laboratory investigations on environmental and clinical samples carried out in IAL (LACEN/SP), which collaborate with the Brazilian Health Ministry for the etiological definition of an outbreak.

MATERIAL AND METHODS

Study characteristics

Between 2018 and 2019 a diarrheal outbreak occurred for around 4 months in Cascavel, PR, Brazil, causing 12,223 diarrhea cases. The Brazilian health authorities investigated the epidemiological and clinical characteristics of this acute diarrheal disease caused in the population during this outbreak.

The local sanitation company investigated the drinking water and confirmed the contamination by protozoa of genera *Cryptosporidium* spp. and *Giardia* spp. in the springs. In addition, symptomatic individuals with diarrhea, living in this municipality, had positive parasitological tests for *Cryptosporidium* spp. and *Giardia* spp. These facts led the health authorities to request IAL to expand the research in the following items:

I. Confirmation of diagnosis in environmental samples. Expansion of investigation analyzing other drinking water collections. Genotypic characterization of identified parasites as differentiation between *C. parvum* and *C. hominis*, to identify the probable infection source.
II. Confirmation of parasitological diagnosis in clinical samples. Quality control on slides using specific stains, which confirmed the protozoa in the initial diagnosis and other protozoa species.

Environmental samples

The health authorities from the municipality sent to IAL 26 water samples packed in 20 L bottles, collected in "Water Treatment Station". Water samples were collected at: i. raw water: untreated water collected in river surface (4 samples); ii. water from the internal washing of filters that were used to filter raw water from springs (5 samples); iii. treated water from the reservoir's surface (4 samples); iv. drinking water from home taps (7 samples); v. drinking water from schools and public hospitals taps (3 samples); and v. water from springs and/or spouts (3 samples).

Clinical samples

At the same time, 47 frozen stool samples from individuals with diarrhea and living in the municipality were sent to IAL to confirm the diagnosis for intestinal coccidia. Previously, these individuals had positive parasitological diagnosis performed in local laboratories. They sent, also, for diagnosis confirmation, 8 slides containing stool smears from symptomatic individuals stained by fuchsin and 10 computed slide images analyzed by local laboratories that identified *Cryptosporidium* spp.

Preparation of environmental samples for analysis

Each water sample (20 L) was filtered, with the aid of a vacuum pump, in a system of hoses coupled in a closed container containing a membrane 142 mm-diameter, 0.22 µm-pores, under pressure of 0.7 kgf/cm^2. Then, the membrane containing the recovered from the filtration was removed from the apparatus, placed in a Petri dish to remove the retained material with the aid of a cell scraper. One part of this material was used for microscopic analysis of parasites and the other (200 µL) for DNA extraction[9].

Preparation of clinical samples for analysis

Frozen fecal samples were inadequate for carrying out the complete protocol for microscopically investigation of intestinal and opportunistic parasites (recommendations of Health Ministry-CGLLAB Manual)[12]. Thus, stool samples were concentrated and purified by flotation centrifuge in sucrose procedure[13]. About 15 g of each stool sample was diluted in distilled water (30 mL), filtered through gauze with 13 threads per cm^2 folded in four and centrifuged 3 times (2400 g for 10 min). After discarding the supernatant, the pellet was suspended in 15 mL of saturated sucrose solution (1200 g/mL) and centrifuged (500 g for 3 min). The supernatant (2 mL) was diluted 10 times in distilled water and centrifuged at 2400 g for 10 min. The sediment was suspended in 1 mL of distilled water and

the solution was used for microscopic examination after specific staining methodologies. An aliquot (200 µL) reserved for DNA extraction.

Parasitological diagnosis

From the water and stool samples concentrated by the sucrose flotation centrifuge methodology, smears were performed on slides. Kinyoun stain was performed for investigation of intestinal coccidia oocysts with alcohol-acid resistant staining characteristics[14]. The investigation of evolutionary forms of *Cryptosporidium* spp. and *Giardia* spp. was determined by direct immunofluorescence using a commercial Kit Merifluor® (Meridian Bioscience Inc., Cincinnati, Ohio). The images were observed in optical and immunofluorescence microscopies. The morphometric analyses and the revisions of slides sent by local laboratories were carried out by captured in a Canon A640 digital camera and treated by the AxioVision 4.8.2 program, Zeiss.

Molecular diagnosis

Aliquots of 200 µL of prepared samples (water or stool) were digested for 12-18 hours at 56 ºC with proteinase K (20 µg) in 40 µL of AL buffer (Qiagen), under agitation at 500 oscillations/min. Next, DNA extractions were performed using QIAamp DNA Mini Kit (Qiagen®), following the manufacturer's protocol. The purity concentrations of the DNA samples were determined by O.D. ratio at 260 and 280 nm on a NanoDrop ND1000. DNA samples were diluted with ultrapure water to a concentration of 100 ng/µL for use in PCR.

For conventional PCR (cPCR) and semi-nested PCR (snPCR), amplifications were performed using GoTaq®Green Master Mix kit (Promega), which contain per reaction (12.5 µL) one unit of Taq DNA polymerase, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$ and 200 mM of each deoxynucleotides triphosphates (dATP, dGTP, dCTP, dTTP). In each reaction were added 5 µL of tested DNA and 10 pmoles of each primer in a final volume of 25 µL. Amplifications were performed using a Veriti® Thermal Cycler (Applied Biosystems®).

For real-time PCR (qPCR), amplifications were performed in a final volume of 20 µL per reaction. The tested DNA (3 µL at a concentration up to 100 ng/µL) or the control DNA (5 ng/µL) were added to the solution containing 10 µL of 2X TaqMan Universal PCR Master Mix and 1.25 µL of primer set containing 18 µM of each molecular marker and 5 µM of the probe. The positive control and negative control (ultrapure water) were included in all reactions and amplifications were performed in the ABI 7500 Real Time PCR System (Applied Biosystems) equipment.

The determination of *Giardia* spp. was performed by single-tube snPCR. Initially, 18 µM of each primer were added to each reaction: GG-F (5'AAGTGCGTCAACGAGCAGCT3'), G7-F (5'AAGCCCGACGACCTCACCCGCAGTGC3') and G759-R (5'GTCGTCTCGAAGATCCAGGGCGGCCTC 3')[15-17]. Amplifications were performed with a program consisting of 20 cycles (94 °C for 30 sec; 65 °C for 30 sec and 72 °C for 30 sec) for annealing (G7-F and G759-R). This was followed with an interval of 4 °C for 2 min. Then, 35 more cycles were added to the program (94 °C for 30 sec; 58 °C for 30 sec and 72 °C for 45 sec) for annealing the primers GG-F and G759-R[15].

The detection of *Cryptosporidium* spp. was performed by cPCR using 18 µM of each primer: CryIAL1F

(5'TACCTACGTATGTTGAAACTCCG3'), CryIAL2F (5'AGGATACGAAATATCAGGAAAGC3') and CryIAL3-R (5'TGTATATCCTGGTGGGCAGACC3'), which amplify a specific sequence of a gene that encodes an oocyst wall protein from *Cryptosporidium* spp.[17]. In each reaction was used 0.1 µL, 0.5 µL and 0.5 µL of CryIAL1F, CryIAL2F and CryIAL3R respectively. Amplifications were performed in an initial step consisting of 20 cycles (94 °C for 30 sec; 61 °C for 30 sec and 72 °C for 30 sec) for annealing the external molecular primers CryIAL1F and CryIAL3R. After the first step, the second step was followed with 35 cycles (94 °C for 30 sec; 56 °C for 30 sec and 72 °C for 45 sec) for annealing the primers CryIAL2F and CryIAL3R[17].

The discrimination of the most common species in epidemic outbreaks, *C. parvum* and *C. homini*, was determined by qPCR, using 18 µM of each of the primer set. For *C. parvum*, the primers were JVAF (5'ATGACGGGTAACGGGGAAT3'), JVAR (5'CCAATTACAAAACCAAAAAGTCC3') and the FAM-labeled probe (5'ATTTATCTCTTCGTAGCGGCG3'), which amplifies a sequence from the 18S rRNA region of *C. parvum*. For *C. hominis*, the primes were JVAGF (5'ACTTTTTGTTTGTTTTACGCCG5'), JVAGR (5'AATGTGGTAGTTGCGGTTGAA3') and the probe labeled "FAM" (5'ATTTATTAATTTATCTCTTACTTCGT3')[18].

Molecular identification of *C. cayetanensis* was performed using 18 µM of each of the primer: Cyclo250F (5'TAGTAACCGAACGGATCGCATT3'), Cyclo350R (5'AATGCCACGGTAGGGCAATA3') and the probe labeled with "FAM" Cyclo281 (5'CCGGCGATAGATCATTCAAGTTTCTGACC 3')[19]. qPCR amplifications were performed in an initial cycle of 50 °C for 2 min, a cycle at 95 °C for 5 min and then 45 cycles at 95 °C for 30 sec and at 67 °C for 30 sec.

The amplified cPCR products were separated by electrophoresis in a horizontal electrophoretic system in agarose gel 1.2%, TBE buffer (45 mM Tris-Borate and 1 mM EDTA pH 8.0), containing 0.5 ethidium bromide g/mL. The molecular mass marker with multiple fragments of 100 bp was added in all gels. The runs were performed at 100 Volts for 1 h. Samples were visualized on a Gene Genius transilluminator (Gel Capture Pro Program, version 4.5.3) ultraviolet at a wavelength of 302 nm[17].

RESULTS

Out of the 26 water samples analyzed by molecular and parasitological methods, 8 (30.8%) had waterborne protozoa. Of these, 1 was determined by direct immunofluorescence, 4 by PCR, 2 by parasitological diagnosis and 1 by both methods (parasitological and PCR). Among the positive samples, the most frequent protozoan was *C. cayetanensis* detected in 4 samples (15.5%). *C. hominis* was detected in 3 samples (11.5%). Only 1 (3.8%) water sample from backwash filters of the municipal treatment plant was positive for *Giardia* spp. **Table 1** shows, in detail, the results of each water sample studied.

The 47 frozen stool samples sent to IAL, the local laboratory determined that 34 were positive for *Cryptosporidium* spp., one for *Cyclospora cayetanensis* and 12 had non-pathogenic parasites. Likewise, in review of eight slides and 10 microphotographs, no structures suggestive of these intestinal coccidia were found. The single positive case for *C. cayetanensis*, determined in local laboratories, was also confirmed in IAL by the parasitological and molecular methods. The other non-pathogenic parasites found were not confirmed in IAL. **Figures 1A**, **1B**, **1C** and **1D** show *C. cayetanensis* oocysts stained with Carbol-Fuchsin solution according to Kinyoun method isolated from a water sample and a stool sample, respectively.

Table 2 presents, in detail, the results of each stool sample. From the 47 stool samples, 11 (23.4%) were positive for protozoa. Of these, *Giardia* spp. was detected in 3 samples by direct immunofluorescence (**Figures 1E** and **1F**), 8 were positive for *C. cayetanensis*.

Regarding the age group, the 21 stool samples from individuals aged between 0 to 29 years old, 2 (9.5%) were positive for *C. cayetanensis* and 3 (14.3%) for *Giardia* spp. Out of the 20 individuals aged 30 to 59 years, 4 (20%) were positive for *C. cayetanensis*. The 6 stool samples from individuals aged over 60 years, 2 (33.3%) were positive for *C. cayetanensis*. Contrarily from water samples, in stool samples, *Cryptosporidium* spp. was not shown as suspected the local laboratory.

DISCUSSION AND CONCLUSION

According to the recommendations of the Brazilian Health Ministry, water quality intended for human consumption, both surface and underground, must have physical, chemical and biological

Table 1. Analyses on water samples collected in the diarrheal outbreak

Water collection points	Number of samples	Molecular and Microscopic Diagnosis					
		Giardia spp.		*Cryptosporidium hominis*		*Cyclospora cayetanensis*	
		Neg	Pos	Neg	Pos	Neg	Pos
River surface (Untreated)	04	04	0	03	**01**[a]	02	**02**[b]
Backwash (Untreated)	05	04	**01**[c]	03	**02**[a]	04	**01**[a]
Reservoir surface Treated)	04	04	0	04	0	03	**01**[a,b]
Home faucets	03	03	0	03	0	03	0
Public faucets from schools and hospitals	07	07	0	07	0	07	0
Fountains/spouts	03	03	0	03	0	03	0
Total	**26**	**25**	**01**[c]	**23**	**03**[a]	**22**	**04**[a,b]

Neg (negative) or Pos (positive) diagnosis performed by molecular [a], parasitological [b] or direct immunofluorescence methods [c]

Table 2. Analysis on stool samples collected from individuals with symptoms caused during the diarrheal outbreak

Stool samples/ Age group	Number of samples	Molecular and Microscopic Diagnosis					
		Giardia spp.		*Cryptosporidium* spp.		*Cyclospora cayetanensis*	
		Neg	Pos	Neg	Pos	Neg	Pos
0 to 29 years old	21	18	**03**[c]	21	0	19	**02**[a]
30 to 59 years old	20	20	0	20	0	16	**04**[a,b]
Up to 60 years old	06	06	0	06	0	06	**02**[a,b]
Total	**47**	**44**	**03**[c]	**47**	**0**	**41**	**08**[a,b]

Neg (negative) or Pos (positive) diagnosis performed by molecular [a], parasitological [b] or direct immunofluorescence methods [c]

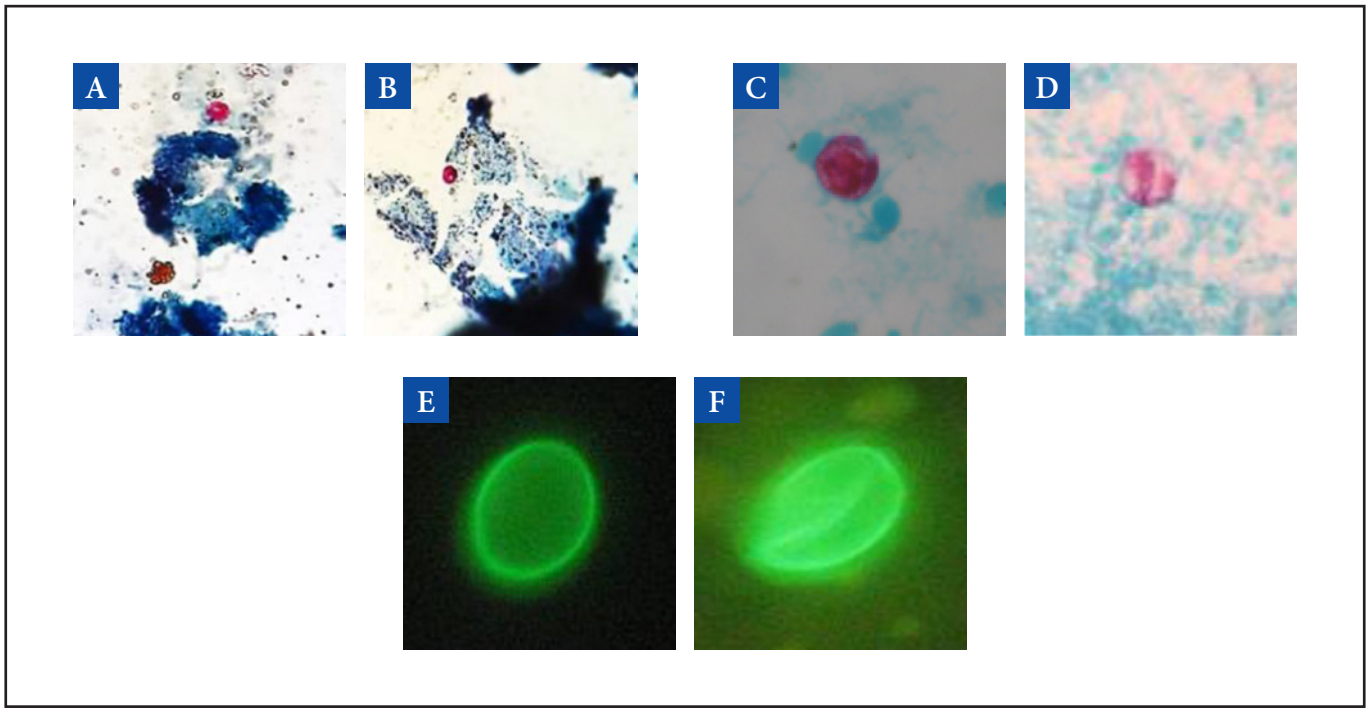

Figure 1. Microscopic images of *Cyclospora cayetanensis* oocysts isolated from water sample (A and B – 400X magnification) and from feces sample (C and D – 1000X magnification), stained with fuchsin (Kinyoun technique); cysts of *Giardia* spp. isolated from feces sample, stained by direct immunofluorescence (E and F – 1200X magnification)

characteristics in accordance with world quality standards[20-22]. When these standards are not respected, serious health problems occur in populations, especially in the transmission of pathogenic protozoa, causing important morbidity and mortality rates[23-25]. The protozoa analyses in water bring a series of scientific and technological challenges, since the internationally recognized methodologies are highly complex and the matrices are complex[26,27]. Brazil has one of the largest potential reservoirs of continental water, including surface and groundwater, but, the population growth, disorderly urbanization and industrial development have compromised water resources. Such overloads resulted in deficiencies in the public sanitation system, increasing the risk of waterborne diseases. The transmission of protozoa is favored by high rainfall, flooding and overflowing septic tanks. Such factors are allied to dairy, livestock, swine and poultry production activities, which cause a large waste volume[28,29].

These aggravating factors can be seen in the outbreak reported here. Samples collected in the backwash filters presented simultaneously the three researched protozoa denoting a concentration of 30.8% of pathogens during the water filtration process. Out of the stool samples analyzed, 33% contained protozoa[28,29].

Regarding the age group of those affected, according to registry data, it ranged from 10 months to 87 years old. The fact that most of the infected people were adults, led us to suspect that the local findings showing *Cryptosporidium* could be confused with *C. cayetanensis*, since this parasite affects a higher age group. Previous studies have also reported clinical cases of diarrhea at older ages, such as, around 13% of foreign adults were infected with *C. cayetanensis* in Indonesia, highlighting the migration capacity of this protozoan[30]. In Brazil, the first reported outbreak caused by *C. cayetanensis* occurred during 1999 to 2000 in a city with around 10 thousand inhabitants (General Salgado, SP)[31]. At that time, 275 cases were

reported, with 66.6% positivity in the analyzed stool samples. Although water samples of the region were not analyzed, it was suggested that water was the source of contamination, due to the large number of cases and extensive dispersion of affected people. A similar case occurred in Antonina, PR city after identification of *C. cayetanensis* in stool samples. About 600 cases of diarrhea were reported and associated with the consumption of contaminated water, resulting from the distribution pipe rupture in the city. Although the protozoa species was not investigated in water, the evidence suggested contamination by water, due to the expressive number of affected people[32]. The protozoa detection in water samples is hampered due to the amount of sample collected for analysis, whether sample water is raw or filtered, independent of its turbidity[33-35]. However, in outbreaks, the monitoring of environmental samples is extremely important for the elucidation of the origin of the contamination and control measures by authorities[36].

Cryptosporidium spp., *Giardia* spp. and *C. cayetanensis* are a constant concern for water producers and the food industry, as these parasites are transmitted via the fecal-oral route and are responsible for different outbreaks of gastrointestinal diseases. Normally, these outbreaks are caused by the consumption of contaminated water, treated or not. Different factors contribute to the wide dispersion of these protozoa in the environment. The most important of them is the (oo) cysts resistance, which remains infective in surface water for more than six months at a temperature of 20 °C and after chlorination treatment[37].

The first suspect was that causative of this outbreak was *Cryptosporidium* spp., since new and relapses cases appeared. The second suspect was that the outbreak must have occurred through water transmission, since it was dispersed in different municipality neighborhoods, whose water source was common among those people affected. Thus, the water and stool samples of this locality were sent to IAL. Among these samples were included those of backwash water from treatment filters aiming to investigate protozoa that accidentally could have entered through the treatment system and affected the population. Exactly in these backwash products were found, simultaneously, positive samples for *C. cayetanensis*, *C. hominis* and *Giardia* spp., indicating the presence of these pathogens during the process of water treatment. *C. hominis* occurrence in these samples suggests the spring contamination with human waste brought by the sewage. Among samples of treated water and ready for consumption (collected after treatment) only *C. cayetanensis* was detected.

After a suspected outbreak, health authorities of the municipality formed a commission responsible for conducting the investigation. They requested from the "Sanitation Company" a plan to recover the financial damages incurred by the municipality during the outbreak, with the proposal of investments to invest in Health actions in the municipality. The amount may be corresponding to the loss and commitment to making advances in protozoa detection in water. According "Municipal Health Department", the outbreak caused big financial loss for the municipality, since during the outbreak, 12,223 individuals with diarrhea were treated. On the other hand, a total of 12,189 patients were attended in 2017 and 13,689, in 2018. Thus, during the outbreak, individuals with diarrheal were attended three times more than patients attended in other periods. These financial values for care and treatments could have been applied to the municipal health network in general, where the population ended up being affected directly or indirectly by water contamination. IAL issued reports on all the analyses carried out, including the detailed steps of the methodologies and photomicrographs of the findings with images at different magnifications, also providing the local network of laboratories, reference material containing stained slides for study and comparison.

Finally, the protozoa investigation in water samples by laboratory methodologies is unusual in Brazil. The parasitological analysis includes complementary methodologies, such as staining, molecular analyses, differential diagnosis, adequate training and ability of professionals in order to evidence and differentiate the parasitic forms. In addition, they must distinguish these forms from artifacts and other structures. These factors cause high cost of these methodologies and the difficulties in collecting samples to be analyzed during the outbreak.

The introduction of such methodologies in the network of Public Health Laboratories can favor the quick elucidation of epidemic outbreaks. Thus, rapid actions could be determined to alternative measures of water supply for the population. The elucidation of this epidemic outbreak, showing multiple pathogens in the different matrices analyzed, denotes the importance of a multiple disciplinary approach and the interaction between the laboratories of the network in support of the differential diagnosis.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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The authors did not declare.

AUTHORS' CONTRIBUTION

The authors declare that all contributed equally to this work and approved the final version.

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