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SHORT COMMUNICATION

OCCURRENCE OF PARASITES IN SALADS IN RESTAURANTS IN APARECIDA DE GOIÂNIA, GOIÁS, BRAZIL

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ABSTRACT

Parasitic diseases are the most widespread diseases in the world. They are transmited via contaminated water or food. Considering that the daily consumption of vegetables is estimated at 142g per person, the purpose of this study was to evaluate the occurrence of parasites in salads available for consumption in restaurants in Aparecida de Goiânia, Goiás State. Salad samples were collected from the restaurants and parasitological analysis was performed using the Willis, Hoffman, Faust and Ziehl Neelsen techniques as well as cultures for the isolation of free-living amoebae. 51 samples were analyzed, 16 (31.4%) were positive. The parasites detected were: *Acanthamoeba* spp. in 12 (23.5%); free-living larvae, *Schistosoma mansoni* and *Entamoeba coli* in 1 (2.0%); *Endolimax nana* in 2 (3.9%). The PCR technique determined that 17.6% of the samples presented *Toxoplasma gondii* DNA. These techniques evidenced that the salad samples presented parasite contamination not only in the restaurants with the lowest price per Kg, but also in the most expensive ones. Therefore, in addition to effective sanitary surveillance, prophylactic measures are necessary regarding suppliers, handlers and restaurant owners to prevent the spread of these and other parasites.

KEY WORDS: Food contamination; food parasitology; polymerase chain reaction.

Parasitic diseases are the most common in the world, affecting approximately one quarter of the world's population. They are among the most aggravating public health problems, especially in developing countries, being widespread and highly prevalent, due to precarious living conditions and insufficient income. Their transmission depends on factors such as basic sanitation and adequate hygiene habits (Santos et al., 2017).

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There are several parasites transmitted through food and water contaminated by cysts of protozoa and / or helminth eggs, which can infect humans. The consumption of vegetables is essential in a healthy diet since these are an important source of vitamins, minerals and fibers. Vegetables, especially those consumed *in natura*, are extremely important in public health because they are widely consumed by the population. Due to the use of irrigation water contaminated with human feces and the use of organic fertilizer containing fecal waste, as well as the vegetables being exposed to birds, flies and mice, these may contain parasites serving as an important means of enteroparasitoses transmission (Nogueira et al., 2016).

The Brazilian vegetable trade plays an outstanding part in the economy having contributed R$ 5.5 billion to the country in 2013. In addition, the daily consumption of vegetables reached 142 grams *per capita* in 2009, according to data from the Food and Agriculture Organization (FAO), the United Nations Organization agency for agriculture. The intense flow within the vegetable trade neglects previous hygiene measures of these foodstuffs in regard to commercialization or consumption. Generally, parasitic diseases are due to this neglect in public and individual health, ignoring quality control practices from cultivation in vegetable gardens, transportation, marketing in retail chain stores or street markets and consumption in homes (Franco, 2013).

There is still no information on the prevalence of parasites found in restaurant salads in the city of Aparecida de Goiânia. Therefore, this study monitored the contamination of vegetables sold in some restaurants in the city by collecting salad samples, providing data of interest for both public health and health surveillance regarding the situational diagnosis concernig hygiene of these foodstuffs.

The salads were picked randomly from 51 restaurants, directly from the self-service counters, in the City of Aparecida de Goiânia, Goiás, and examined on the same day, totaling 51 samples. The vegetables were first washed in containers with distilled water and neutral household detergent using brushes to remove dirt. After washing, the liquid was strained with the aid of gauze folded four times and poured into chalices to perform the other procedures. Five different parasitological techniques were used: Willis, Hoffman, Faust, Ziehl Neelsen methods and culture for the isolation of free-living amoebas (FLA).

Willis' technique aims to isolate eggs and larvae of some types of nematodes and protozoan oocysts by spontaneous fluctuation using a low specific density saturated sodium chloride (NaCl) solution, and is based on the property that eggs have of floating to the surface, then adhering to a slide placed on top of the liquid. The materials used are: saturated NaCl solution, wide mouth container, blades, coverslips, lugol and disposable cups. In distilled water, NaCl is added until the excess salt no longer dissolves in the solution (in the proportion of 400g NaCl to 1L of water). In the preparation of the saturated

solution, table salt is used. The procedure consisted of: washing the salads with distilled water and detergent, using disposable brushes on the leaves (one for each sample), then putting a quantity of this sieved water in a disposable cup containing ¼ of its capacity of saturated NaCl solution; this saturated saline solution was suspended until total homogenization; the slide was then placed on the edge in contact with the solution for five minutes; then the slide was removed and the lugol and cover slip were put on (Willis et al., 1921).

The Hoffman-Pons-Janner method, also called spontaneous sedimentation or Lutz method, is based on spontaneous sedimentation in water. It is used to detect heavy helminth eggs, when sedimentation remains for a period of at least two hours, and for protozoan-free cysts, remaining for a period of 24h (Hoffman et al., 1934).

Faust's technique is utilized to research protozoan cysts and light helminth eggs. The centrifugal-flotation method is based on the principle of specific density difference between helminth eggs, protozoan cysts and salad materials, so that these organisms float on the surface of reagents with specific density. Centrifugation in Zinc Sulfate ($ZnSO_4$) 33% with a density of 1.18 g / mL was performed. The solution was prepared with 300g of $ZnSO_4$ and 660 mL of distilled water, centrifuged for 10 min at 450 x G at 24 °C. The supernatant liquid was discarded and the pellet was resuspended by centrifuging again, for 10 min at 450 x G at 24 °C (Faust et al., 1938).

The Ziehl-Neelsen technique aimed at detecting sporozoan oocysts, including *Cryptosporidium* spp. oocysts in the salad material. To start the procedure, a container with salad samples or concentrated sediments was used. The materials used were: blade, 10% Kinyoun dye, acid-alcohol solution, 3% aqueous malachite green solution, immersion oil, distilled water, pipettes, basic fuchsin, concentrated sulfuric acid (H_2SO_4) and an oven (Fayer et al., 2000; Henricksen & Pohlenz, 1981).

For the purpose of FLA research, the samples were placed in sedimentation chalices and, after about 24h, the sediments obtained were centrifuged at 450 x G for 5 minutes and sown in Petri dishes containing 1.5% non-nutrient agar, covered with *Escherichia coli* suspension, inactivated by heat. The sowing was carried out in a vertical laminar flow hood. The plates were incubated at 25 ° C (room temperature) and observed for 14 days for the presence of FLA as well as being studied under an inverted optical microscope. The amoebae were identified by morphological criteria already established for the identification of cysts and / or trophozoites (Alves et al., 2012).

The Polymerase Chain Reaction (PCR) was performed in order to detect *Toxoplasma gondii* genetic material. The deoxyribonucleic acid (DNA) was extracted from the sample sediments using the Ausubel et al. (1999) modified chloroform phenol method. 250 µL of each sediment were placed in microtubes with 750 µL of buffer solution and 10 µL of proteinase K and left overnight in an oven at 35 ºC. The samples were then centrifuged, the

supernatant was discarded and 50 µL of deionized water were added and stored at 4 ºC. The primers Toxo-B5 (5'-TGA AGA GAG GAA ACA GGT GGT CG - 3 ') and Toxo-B6 (5'-CCG CCT CCT TCG TCC GTC GTA - 3') were used (Santos et al., 1993) to amplify the sequence from 131 to 191 base pairs of the conserved region of the B1 gene (Franzen et al., 1997).

This reaction required 17.3 µL sterile Mili-Q, 1.0 µL of Magnesium Chloride, 2.5 µL of 10X Buffer (Invitrogen®), 0.2 µL of Taq DNA Polymerase (Invitrogen®), 0.5 mM deoxynucleotides (dATP / dTTP / dGTP / dCTP, Sigma®), 50 pmoles of each reaction primer (Invitrogen®) and 2µL of extracted DNA totaling a final volume of 25µL. For amplification, an initial denaturation occurred at 94 ºC for 5 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 62 ºC for 1 minute and extension at 72 ºC for 1 minute followed by final extension at 72 ºC for 10 minutes. The products amplified by PCR were visualized by electrophoresis with 6% polyacrylamide gel and revealed with silver (Rezende et al., 2019).

The parasitological methods detected that 17 of the 51 samples evaluated (33.4%) were contaminated with parasitic evolutive forms. *Acanthamoeba* spp. in 12 samples (23.5%), *Endolimax nana* in two samples (3.9%), *Schistosoma mansoni* in one sample (2.0%), free-living larvae in one sample (2.0%) and *Entamoeba coli* in one sample (2.0%), as shown in Table 1.

Table 1. List of parasites found in salads sold in restaurants in Aparecida de Goiânia, Goiás State, Brazil.

Parasite	n	%
Acanthamoeba spp.	12	23.5
Free-living larvae	1	2.0
Endolimax nana	2	3.9
Schistosoma mansoni	1	2.0
Entamoeba coli	1	2.0
Negative Samples	34	66.6
TOTAL	51	100.0

Concerning the molecular study, 9 of the 51 samples analyzed were positive for the presence of *T. gondii* DNA, signifying 17.6% of the restaurants.

The restaurants where the salads were collected are located in neighborhoods with basic sanitation, so the contamination does not come from the water in these establishments, since it is treated. It is possible that the vegetables are delivered contaminated and do not undergo effective hygiene methods before they are prepared and served. In 2004 the National Health Surveillance Agency (ANVISA) published Collegiate Board Resolution (RDC) number 206, which provides the technical regulation of good practices

for food services. The standards in this legislation must be followed in order to offer pathogen-free food under suitable hygienic conditions for consumption (Brasil, 2004).

The significant occurrence of *Acanthamoeba* spp. in the samples (23.5%) is due to the fact that this FLA has several habitats, such as air, soil and water and although the ingestion of cysts and / or trophozoites does not present direct disease risks, this protozoan can house other pathogens and work as a vector for other diseases (Maschio et al., 2015; Greub & Raoult, 2004). Therefore, the detection of FLA in food evidences the close contact of the population to these parasites and the possibility of diseases such as keratitis and encephalitis (Castrillón & Orozco, 2013).

This work detected the presence of *Endolimax nana* and *Entamoeba coli*. Although they are commensal, this still indicates that there is contamination of vegetables with fecal specimens and that there is a susceptibility to contamination by other enteroparasites, such as *Entamoeba histolytica* which is pathogenic and leads to a serious infection as it has developed a number of mechanisms of virulence to evade the immune system (Debbie et al., 2020).

Finally, the presence of *Schistosoma mansoni* (2.0%) was noted, corroborating the study by Lima et al. (2018), in which this parasite's eggs were found in 1.2% of vegetable samples from supermarkets in Maceió, Alagoas. In Goiás, there are no previous studies reporting the presence of *S. mansoni* in salads. However, in spite of not being an endemic area, care should be taken as this parasite can cause ascites. Basic sanitation measures together with adequate food hygiene practices may be able to minimize the presence of all the parasites found.

The findings in this study regarding *T. gondii* DNA (17.6%), indicate a serious public health problem once this parasite is able to cause congenital complications (abortion, stillbirth and hydrocephalus in newborns), ocular deficiencies (retinochoroidal lesions leading to chronic ocular disease), encephalitis (in the immunocompromised) and multivisceral toxoplasmosis (Dubey, 2010). A recent study by Marques et al. (2020) showed *T. gondii* DNA (also using PCR technique) in 40.0% of their samples of fresh vegetables and berries from random locations in Portugal and Spain. The authors highlighted that water and food matrices may be accidentally contaminated by environmental oocysts (Shapiro et al., 2019).

A study performed in Mampong-Ashanti, Ghana, by Reynalds et al. (2020) revealed the presence of parasites in 80 of 120 (66.7%) ready-to-eat salads analyzed. *Giardia duodenalis* (24.4%), *Ascaris lumbricoides* (19.2%), *Moniezia* spp. (11.7%), *Entamoeba coli* (5.8%), *Toxocara* spp. (5.8%), *Taenia* spp. (4.2%), *Fasciola hepática* (5.8%), *Enterobius vermicularis* (4.2%), *Trichuris trichiura* (3.3%) and *Toxoplasma gondii* (0.8%). *G. duodenalis* and *A. lumbricoides* were the two most predominant parasites in their study, differently from the present study (*Acanthamoeba* spp.) in which these were

not isolated. Although a higher rate of *T. gondii* was found in this study, this may be explained by the different sensitivity of both methodologies, they did not use PCR (more sensitive), only microscopy.

The present study might have detected higher parasite rates if the straining process had been performed with the gauze folded fewer times (it was folded four times). The gauze may have trapped parasites signifying that the contamination could be even greater.

Regarding the price per kilo, values ranged from R$ 11.99 to R$ 40.00, with R$ 29.99 being the most prevalent as shown in Table 2. The wide variety of prices regarding the contaminated samples reveals that insufficient hygiene measures do not occur only in restaurants where the Kg is cheaper, but also in the most expensive establishments.

Table 2. Relation between parasite prevalence and price in Reals (R$) per Kg in the restaurants where the salad samples were collected in the City of Aparecida de Goiânia, Goiás State, Brazil.

Parasite	Kg(R$)
Negative Samples	11.99-40.00
Acanthamoeba spp.	21.00-33.90
Free-living larvae	29.99
Endolimax nana	29.99
Schistosoma mansoni	21.00-28.99
Entamoeba coli	31.99

This study is the first report on the presence of parasites in samples from salads sold in restaurants in the city of Aparecida de Goiânia, Goiás. It is therefore clear that these salads are probably transmiting a number of parasitoses.

In conclusion, contamination occurs in the most diverse places, among the most expensive and the cheapest establishments. Therefore, adequate inspection and skilled labor are paramount in order to eliminate parasites in salads. Strict sanitary quality control in food hygiene processes by the owners of food establishments is even more essential.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

REFERENCES

1. Alves DSMM, Moraes AS, Nitz N, Oliveira MGC, Hecht MM, Gonçalves RG, Cuba CAC. Occurrence and characterization of *Acanthamoeba* similar to genotypes T4, T5, and T2/T6 isolated from environmental sources in Brasília, Federal District, Brazil. *Exp Parasitol 131:* 239-244, 2012.
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman, JG, Smith JA, StruhlK. *Short protocols in molecular biology.* Wiley Sections: New York, 1999.
3. Brasil. Agência Nacional de Vigilância Sanitária. *Resolução nº 216 de 15 de setembro de 2004.* Dispõe sobre Regulamento Técnico de Boas Práticas para Serviços de Alimentação. 2004. Available in: http://portal.anvisa.gov.br/documents/33916/388704/RESOLU%25C3%2587%25C3%2583O-RDC%2BN%2B216%2BDE%2B15%2BDE%2BSETEMBRO%2BDE%2B2004.pdf/23701496-925d-4d4d-99aa-9d479b316c4b. Accessed at: 03/20/2020.
4. Castrillón JC, Orozco LP. *Acanthamoeba* spp. como parásitos patógenos y oportunistas. *Rev Chilena Infectol 30*: 147-155, 2013.
5. Debbie AS, Chien CH, Shannon M. *Entamoeba histolytica. In*: Ryan ET, Hill DR, Solomon T, Endy T, Aronson NE. *Hunter's tropical medicine and emerging infectious diseases*. Elsevier. Ottawa, 2019. 1264p.
6. Dubey JP. *Toxoplasmosis of animals and humans*. CRC Press: New York, 2010. 313 p.
7. Faust EC, D'Antoni JS, Odom V, Miller MJ, Peres C, Sawitz W, Thomen LF, Tobie J, Walker JH. A critical study of clinical laboratory technics for the diagnosis of protozoan cysts and helminth eggs in 41 feces. *Am J Trop Med Hyg 18*: 169-183, 1938.
8. Fayer R, Morgan U, Upton SJ. Epidemiology of *Cryptosporidium*: transmission, detection and identification. *Int J Parasitol 30*: 1305-1322, 2000.
9. Franco L. *Comércio de hortaliças rende R$ 5,5 bilhões em 2013*. 2013. Available in: https://revistagloborural.globo.com/Noticias/Agricultura/Hortifruti/noticia/2013/12/comercio-de-hortalicas-rende-r-55-bilhoes-em-2013.html. Accessed at: 11/08/2019.
10. Franzen C, Altfeld M, Hegener P, Hartmann P, Arendt G, Jablonowski H, Rockstroh J, Diehl V, Salzberger B, Fätkenheuer G. Limited Value of PCR for Detection of *Toxoplasma gondii* in Blood from Human Immunodeficiency Virus-Infected Patients. *J Clin Microbiol 35:* 2639-2641, 1997.
11. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev 17:* 413-433, 2004.
12. Henricksen SA, Pohlenz JFL. Staining of cryptosporidia by a modifed Ziehl-Neelsen technique. *Acta Vet Scand 22*: 594-596, 1981.
13. Hoffman WA, Pons JA, Janer JL. The sedimentation concentration method in *Schistosoma mansoni. PR J Public Health Trop Med 9*: 283-291, 1934.
14. Lima AF, Santos SMT, Soares BLP, Maria RAR, Junior AFSX. Isolamento e identificação de parasitas encontrados nas verduras dos principais supermercados de Maceió/AL. *Cad Grad Ci Biol Saúde 4:* 47, 2018.
15. Marques CS, Sousa S, Castro A, Costa JMC. Detection of *Toxoplasma gondii* oocysts in fresh vegetables and berry fruits. *Parasites Vectors 13*: 1-12, 2020.
16. Maschio VJ, Corção G, Rott MB. Identification of *Pseudomonas* spp. as amoeba-resistant microorganisms in isolates of *Acanthamoeba. Rev Inst Med Trop Sao Paulo 57*: 81-83, 2015.
17. Nogueira VDF, Caseiro MM, Gagliane, LH. Detecção de estruturas parasitárias em hortaliças comercializadas nos municípios de Santos e São Vicente - SP - Brasil. *Rev Unilus Ens Pesq 13:* 79-85, 2016.

18. Reynalds PKA, Yar DD, Gyamerah I, Apenteng OY, Sakyi S. Fresh Vegetables and Ready-to-eat Salads: Sources of Parasitic Zoonoses in Mampong-Ashanti, Ghana. *European J Nutr Food Saf 12*: 47-55, 2020.

19. Rezende HHA, Lima JAS, Gomes-Junior AR, Melo JO, Storchio HR, Gomes TC, Vinaud MC, Castro AM. Detection of DNA and anti-*Toxoplasma gondii* antibodies in errant cats (*Felis catus domesticus*, Linnaeus, 1758) captured by the Zoonoses Control Center of Goiânia, State of Goiás, Brazil. *Rev Biol Neotrop 16*: 84-88, 2019.

20. Santos FR, Pena SDJ, Epplen JT. Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. *Hum Genet 90*: 655-656, 1993.

21. Santos PHS, Barros RDCS, Gomes KVG, Nery AA, Casotti CA. Prevalência de parasitoses intestinais e fatores associados em idosos. *Rev Bras Geriatr Gerontol 20:* 244-253, 2017.

22. Shapiro K, Oliveira LB, Díxon B, Dumètre A, Wit LA, VanWormer E, Villena I. Environmental transmission of *Toxoplasma gondii*: Oocysts in water, soil and food. *Food Waterborne Parasitol 12:* 1-18, 2019.

23. Willis HHA. A simple levitation method for the detection of hookworm ova. *Med J Aust 8:* 375-376, 1921.