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PARASITE CONTAMINATION AND DETECTION OF *CRYPTOSPORIDIUM* SPP. BY REAL-TIME PCR IN VEGETABLES SOLD IN BULANCAK OPEN-AIR MARKET IN GIRESUN PROVINCE

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Abstract

In the present study, 192 vegetable samples were collected from the open-air market by random sampling method between September 2019 and September 2020. The samples were analyzed by native-lugol, sedimentation, trichrome, modified trichrome, modified Kinyoun acid-fast and PCR methods. Data were given as percentage and mean. Descriptive statistics of the data set were expressed as mean ± standard deviation (SD). The differences between the means were compared with the Pearson's Chi-Square, Kruskal–Wallis and Likelihood Ratio Chi-Square test. P value of ≤ 0.05 was considered statistically significant.

Parasites were found in all collected vegetables and *Cryptosporidium* spp. (32.29%), *Entamoeba coli* (19.27%), *Ascaris* spp. (12.50%), *Giardia intestinalis* (9.38%), and *Toxocara* spp. (4.69%) were detected at the highest rate as a result of the analysis.

As a result of the study, the presence of parasites was detected in ready-to-eat vegetables collected from Bulancak open-air market in Giresun province. Parasites can be transmitted by eating vegetables that are undercooked, uncooked, or not well washed. In this direction, it can be recommended to take

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effective washing measures before consuming fresh vegetables, to apply raw fertilizer and to educate the public about their application in order to prevent parasitic infection that may arise from contaminated vegetables.

Key words: parasites, public health, food hygiene, vegetables, Black Sea region

Introduction. The contamination of raw vegetables is related to the use of sewage-contaminated and unrefined water sources for irrigation purposes, the post-harvest processing phase and the applications during the consumption phase [1]. Eating undercooked or uncooked infected vegetables and fruits is one of the ways of transmission of gastrointestinal system and tissue parasites [2]. Vegetables consumed especially as salads are important modes of parasite transmission and have been reported to be the cause of foodborne outbreaks in developing countries [3,4].

People's socio-economic status and knowledge level, climate changes, fertilizer selection (use of human and animal fertilizers), inappropriate farming, situations such as transportation, storage and preparation for domestic consumption are effective in the contamination of plants with parasites [5].

In the source information reached, no research has been found on parasites that can be found in vegetables in and around Giresun province. In this direction, it was aimed to evaluate the parasite contamination level of fresh vegetables collected from the open-air market in Bulancak, Giresun, Turkey by direct staining and PCR.

Material and methods. ***Sample collection.*** From the open-air market in Bulancak district of Giresun province, 192 vegetable samples of 16 different types (32 spring, 75 summer, 36 autumn and 49 winter) were collected with random sampling method on a regular basis every two weeks. The number of samples collected varies according to the season and availability. A total of 192 samples were collected and these were; lettuce (*Lactuca sativa*) ($n = 25$), parsley (*Petroselinum crispum*) ($n = 25$), black cabbage (*Brassica oleracea* L. var. *acephala*) ($n = 23$), mint (*Mentha*) ($n = 17$), green onion (*Allium cepa*) ($n = 14$), purslane (*Portulaca oleracea*) ($n = 13$), sugar beet leaf (*Beta vulgaris saccharifera*) ($n = 10$), dill (*Anethum graveolens*) ($n = 13$), grass lily (*Ornithogalum umbellatum*) ($n = 13$), arugula (*Eruca sativa*) ($n = 9$), nettle (*Urtica*) ($n = 9$), cress (*Lepidium sativum*) ($n = 5$), turnip leaf (*Brassica camperstris* subsp. *rapa*) ($n = 4$), ground elder (*Aegopodium podagraria* L.) ($n = 4$), spinach (*Spinacia oleracea*) ($n = 4$) and sarsaparilla (Saparna) ($n = 4$). Each of the samples was placed in clean refrigerator bags, tightly closed, and brought to the Parasitology Laboratory in Faculty of Medicine of Ordu University on the same day.

The collected samples were prepared by the method of Choi and Lee [6] and analyzed by the native-lugol, sedimentation, trichrome, modified trichrome and modified Kinyoun acid-fast methods. Native-lugol method, for the detection

of trophozoites, cysts, eggs and larvae of parasites, sedimentation method, multiplexing of cysts and eggs of parasites by amplification, trichrome stain, species differentiation of protozoa, modified trichrome, diagnosis of microsporidia, modified Kinyoun acid-fast *Cryptosporidium* spp., *Cyclospora cayetanensis* and *Cystoisospora belli* used in the diagnosis.

Polymerase Chain Reaction (PCR) method was applied for the detection of *Cryptosporidium* species in which contamination with vegetables has been frequently reported.

Polymerase Chain Reaction (PCR) method. DNA isolation from all samples was performed using a commercial DNA isolation kit (QIAGEN-QIAamp DNA Stool Mini Kit). Before isolation, the samples were frozen and thawed 5 times, at −80 °C for 30 min and at 56 °C for 10 min. The kit protocol was applied without any modification.

PCR amplification of Cryptosporidium 18 S SSU rRNA locus. PCR study was performed according to the protocol of Xiao et al. [7]. One µl of DNA was used in the I. PCR reaction. Before PCR reaction, the thermal cycler was kept open for 30 min and "hot start" was applied. PCR conditions were set as initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55—58 °C for 45 s, extension at 72 °C for 60 s, and final extension at 72 °C for 7 min. The expected product size as a result of I. PCR was 1325 bp.

F1: 5'- TTCTAGAGCTAATACATGCG-3' and

R1: 5'- CCCATTTCCTTCGAAACAGGA-3'.

I. PCR Master Mix contained 4 µL of 5X FIREPol® Master Mix (12.5 mM $MgCl_2$), 0.5 µL F primer, 0.5 µL R primer, 1 µL DNA and the total volume was adjusted to 20 µL by adding 14 µL of deionized water.

F2: 5'- GGAAGGGTTGTATTTATTAGATAAAG,

R2: 5'- CTCATAAGGTGCTGAAGGAGTA-3'.

Of I. PCR product, 2 µl was used in the II. PCR reaction. PCR reaction conditions were set as 3 min start at 94 °C, 35 cycles (45 s at 94 °C, 45 s at 58 °C, and 60 s at 72 °C) with the final phase at 72 °C for 7 min. Positive samples are expected to form a final product of 823 bp. PCR products were stored at −20 °C.

Gel electrophoresis and imaging. PCR products were run on a 1% agarose gel and the results were evaluated. The gel stained with EtBr was photographed under UV light.

Data analysis. For data analysis, descriptive statistics as a percentage for parasite contamination and identified data of vegetable samples were calculated. The obtained results were presented in the tables as percentages. Descriptive statistics of the data set were expressed as mean ± standard deviation (SD). The differences between the means were compared with the Pearson's Chi-Square, Kruskal–Wallis and Likelihood Ratio Chi-Square test. A p value of ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SPSS v26 (IBM Inc., Chicago, IL, USA) and Minitab 19 (Minitab Inc., State

College, PA, USA) statistical softwares.

Results. According to the data obtained in the study, as a result of Likelihood Ratio Chi-Square Test; No significant correlation was found between parasite positivity by months in both 2019 and 2020 ($p = 0.565$; $p = 0.323$). Considering all of the two-year data, no significant correlation was found between parasite positivity by months ($p = 0.323$). Again, as a result of the Pearson's Chi-Square Test, made regardless of the month; No significant correlation was found between parasite positivity and year ($p = 0.811$). However, the number of detected parasites was compared with the Kruskal–Wallis Test and a significant difference was found between them ($p = 0.001$). Different vegetables were determined by Dunn's multiple comparison test and the results are shown in letters (Table 1).

There was a significant difference between dill and lettuce, onion, turnip leaf and parsley. This can be explained as the parasite contaminations in lettuce, onion, turnip and parsley were significantly higher than in dill.

T a b l e 1

Comparison of parasite numbers according to vegetables

Vegetable	n	Mean	Std. Dev	Mean Rank	Median	IQR	Min.	Max.
Dill (*Anethum graveolens*)	13	0.77	0.83	47.0**B**	1.0	1.5	0.0	2.0
Nettle (*Urtica*)	9	1.33	0.87	73.7 **AB**	2.0	1.5	0.0	2.0
Spinach (*Spinacia Oleracea*)	4	1.25	0.50	66.3 **AB**	1.0	0.8	1.0	2.0
Black cabbage (*Brassica oleracea L. var. acephala*)	23	1.61	1.27	81.9 **AB**	1.0	1.0	0.0	5.0
Lettuce (*Lactuca sativa*)	25	2.52	1.33	121.7 **A**	2.0	1.0	0.0	5.0
Parsley (*Petroselinum crispum*)	25	2.32	1.41	114.9 **A**	2.0	1.5	0.0	6.0
Sarsaparilla (Saparna)	4	1.50	1.29	81.9 **AB**	1.5	2.5	0.0	3.0
Ground elder (*Aegopodium podagraria* L.)	4	2.00	1.15	104.0 **AB**	2.0	2.0	1.0	3.0
Mint (*Mentha*)	17	1.82	1.07	96.8 **AB**	2.0	2.0	0.0	3.0
Sugar beet leaf (*Beta vulgaris saccharifera* leaf)	10	1.20	0.92	65.9 **AB**	1.0	1.3	0.0	3.0
Arugula	1	1.00	$*$	53.0 **AB**	1.0	$*$	1.0	1.0
Arugula (*Eruca Sativa*)	8	1.50	1.07	80.2 **AB**	1.0	1.8	0.0	3.0
Grass lily (*Ornithogalum umbellatum*)	13	2.00	0.82	104.8 **AB**	2.0	2.0	1.0	3.0
Purslane (*Portulaca oleracea*)	13	2.08	1.38	109.1 **AB**	3.0	2.5	0.0	4.0
Onion (*Allium cepa*)	14	2.57	1.02	128.8 **A**	2.5	1.3	1.0	4.0
Turnip leaf (*Brassica camperstris* subsp. *rapa*)	4	2.75	0.50	142.8 **A**	3.0	0.8	2.0	3.0
Cress (*Lepidium Sativum*)	5	1.00	1.00	58.4 **AB**	1.0	2.0	0.0	2.0
$\boldsymbol{p^*}$				**0.001**				

$*$: Kruskal–Wallis Test; The difference between vegetables without a common letter is significant ($p < 0.05$)

Table 2

Frequency distribution of the lowest parasite contamination among fresh vegetables sold in open-air market of Bulancak, Giresun

Parasite	Number of samples	Contamination rate (%)
Acanthamoeba spp.	3	1.56
Cyclospora cayetanensis	2	1.04
Dicrocoelum dentriticum	6	3.13
Amoeba trophozoites	4	2.08
Trichuris trichiura	3	1.56
Taenia spp.	2	1.04
Fasciola spp.	1	0.52
Hookworm egg	2	1.04
Blastocystis spp.	4	2.08
Iodamoeba bütschlii	1	0.52
Dipylidium caninum	3	1.56
Hymenolepis diminuta	3	1.56
Chilomastix mesnili	2	1.04
Cystoisospora belli	1	0.52

The frequency distribution ratios of the detected parasites are given in Tables 2 and 3.

As a result of the analyses made on vegetable samples, *Cryptosporidium* spp. (32.29%), *Entamoeba coli* (19.27%), *Ascaris* spp. (12.50%), *Giardia intestinalis* (9.38%) and *Toxocara* spp. (4.69%) were determined at the highest rate. In addition, species differentiation of 26 larvae (13.54), 27 mites (14.06%) and 101 protozoa (52.60%) could not be made (Table 3).

Table 3

Frequency distribution of the highest parasite contamination among fresh vegetables sold in open-air market of Bulancak, Giresun

Parasite	Number of samples	Contamination rate (%)
Cryptosporidium spp.	62	32.29
Giardia intestinalis	18	9.38
Mite	27	14.06
Ascaris spp.	24	12.50
Entamoeba coli	37	19.27
Unidentified protozoan	101	52.60
Toxocara spp.	9	4.69
Unidentified larva	26	13.54
Microsporidia species	20	10.42

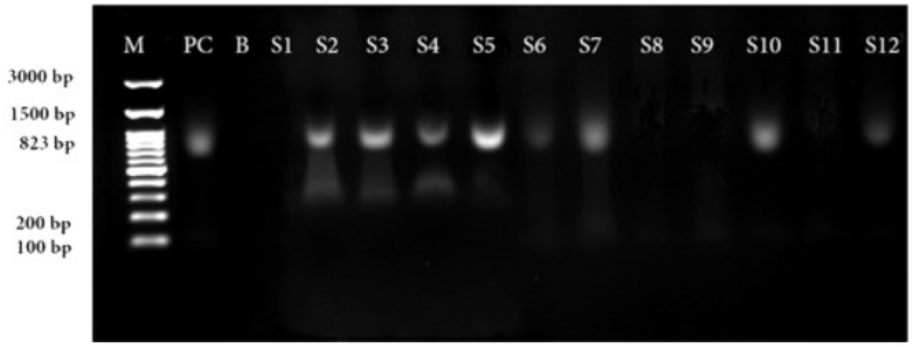

Fig. 1. The results of PCR detected in food samples. Figure 1 shows the results of PCR on the developed gel. M; Marker, PC: Positive Control, B: Blank S1, S8, S9, S11: Negative Samples, and S2-7, S10, S12: Positive Samples were seen in the gel image

According to PCR results for the detection of *Cryptosporidium* spp., positivity was detected in 62 (32.29%) samples (Fig. 1).

Parasitic contamination was found in all vegetable samples examined. The contaminations of parasites in lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*) and black cabbage (*Brassica oleracea* L. var. *acephala*) were detected higher than in other vegetables.

Discussion. Due to the external structure of vegetables, they can be a source of transmission of enteric viruses, bacteria and parasites. In case they are undercooked or uncooked, sources of infection can be transmitted. It has been reported that parasites have been detected in raw vegetables [3]. In the source information reached, no research was found on the transmission of the parasite in vegetables in and around Giresun province. For this purpose, vegetables sold in Bulancak open-air market in Giresun province were evaluated in terms of parasite contamination. In this study, 192 samples from 16 different types of vegetables were collected and examined. As a result of the analysis performed on vegetable samples, it can be stated that *Cryptosporidium* spp. (32.29%), *Entamoeba coli* (19.27%), *Ascaris* spp. (12.50%), *Giardia intestinalis* (9.38%) and *Toxocara* spp. (4.69%) were determined the most. In addition, species identification of 26 larvae (13.54%), 27 mites (14.06%) and 101 protozoa (52.60%) could not be made. In studies conducted in different regions, Sunil et al. [3] found helminth eggs in 3 (2.7%) of 112 raw vegetables sold in open-air market in Mannuthy, Thrissur region of Kerala state, India. Siyadatpanah et al. [8] determined parasite contamination in 200 vegetable samples (spinach, cress, parsley, lettuce, mint, radish, green onion, basil, coriander and green onion) they collected. Nazemi et al. [4] detected *Giardia* spp. and *Entameba coli* in 46 of 92 vegetable samples (leek, coriander and basil) collected from Shahroud, Semnan. On the other hand, Matini et al. [9] found pathogenic (*Giardia intestinalis*, *Toxocara* spp., *Fasciola* spp., *Dicrocoelium*

dendriticum and *Taenia* spp.) in 32 of 383 vegetable samples and non-pathogenic parasites (*Entamoeba coli* and free-living larvae) in two of them. In the study, free larvae without species identification were determined.

El Bakri et al. [10] analyzed 218 fresh vegetable samples in terms of parasite contamination, and protozoan cysts and helminth eggs were detected in 10 of 14 different vegetable varieties at a rate of 15.1% (33/218). According to the results of the study, it was stated that the most contaminated vegetables were broccoli (30.0%), radish (29.4%), spring onions (22.9%), mint and chard (20.0% each) while no parasites were detected in fennel, green pepper, arugula, and carrots (0.0%). In addition, it was reported that mono-parasite contamination was determined in chard and tomato while there was more than one parasitic contamination in other vegetable samples. Again, it was specified in a study performed by do Nascimento Ramos et al. [2] that oocysts, cysts, eggs and/or larvae of gastrointestinal parasites were detected in 79% (79/100) of smooth lettuce. In a study conducted by Alhabbal [1] to determine parasitic contamination, helminth eggs and protozoa cysts were found in 31.386% of a total of 137 fresh vegetable samples. The researcher stated that lettuce and parsley are the dirtiest vegetables. Moreover, Rodrigues et al. [11] investigated a total of 200 fresh vegetable samples from supermarkets and street open-air markets and detected helminth eggs and/or larvae and/or protozoan cysts in 89% (89/100) of lettuce. Similarly, parasites were found in lettuce, parsley, and black cabbage, which is frequently consumed in the region, in 157 of 192 samples.

Li et al. [12] reported that foodborne diarrheal diseases caused by intestinal parasites were an important public health problem worldwide. It has been determined that *Enterocytozoon bieneusi, Cryptosporidium spp., Toxoplasma gondii, Giardia duodenalis, Entamoeba spp., Cyclospora cayetanensis, Blastocystis sp., Cystoisospora belli* and *Balantidium coli* can be found among the protozoan parasites. The results obtained in the present study are similar to the results obtained in the studies in the literature.

Conclusion. In the present study, it was observed that the vegetables collected from Bulancak public open-air market in Giresun province at regular intervals could be contaminated with parasites. Considering this, it was thought that vegetables that are beneficial for healthy nutrition might be infected with contaminated water or animals. Infectious agents can be transmitted to humans as a result of consuming infected raw vegetables without washing them well. For this reason, it can be stated that informing farmers, sellers and consumers about planting, harvesting, transporting, processing and consumption of fruits and vegetables is of great importance in terms of preventing parasitic contamination and protecting public health. In addition, in order to prevent the transmission of infectious agents through vegetables, the people preparing them should be informed about hygiene and sanitation. Control of the use of fertilizers (untreated human and animal waste) can be regulated and Good Agricultural Practices (GAP) (clean

storage, use of clean water) can be applied to reduce the risk of parasitic infections transmitted by vegetables in general. For this reason, it has been concluded that applying hygienic conditions before consumption of vegetables and educating people on this subject may be appropriate in terms of preventing parasitic infections.

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