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Diagnosis Of Contamination Of Vegetables And Fruits With Cryptosporidium Spp Using Nested-PCR Technique

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Abstract:

Cryptosporidium is a parasite that spreads all over the world and is transmitted through food contaminated with the infectious process. In this study, Cryptosporidium parasite was diagnosed on fruits and vegetables based on microscopic diagnosis of isolated samples of vegetables and fruits as well as molecular diagnosis. The aim of this study was to isolate Cryptosporidium parasite from vegetables and fruits and extract its DNA using a (modified) method that can be easily applied in laboratories and then molecularly diagnosed.Where 230 samples were examined microscopically and stained using Zyl Nelson Axial dye, and it was found that there were injuries in only 3 samples of the studied models, with a percentage of 1.30%. DNA was extracted for all samples using laboratory prepared solutions, where 102 samples were obtained from the total samples, with a percentage of 44.3%. The gene HSP-70 was used to molecularly diagnose Cryptosporidium spp and it was found that 10 samples were positive from all studied models with a percentage of 9.80%.

Key words: Cryptosporidium spp, HSP-70 gene, Nested- PCR, contamination of vegetables and fruits.

1- Introduction:

In the past few years there has been a growing interest in the study of food-borne parasites that pose a threat to the public health and economic well-being of society (Devleesschauwer et al., 2017),Several studies have confirmed an increasing rise in the incidence of diseases related to food contamination and the increase in the spread of intestinal parasites, especially in developing countries (Hassan, Farouk & Abdul-Ghani, 2012), as eating raw vegetables is an important means of transmission of many parasitic diseases (El Said Said, 2012),One of these parasites, which is characterized by its wide spread, is Cryptosporidium spp, which is the second most common cause of diarrhea (Bouzid, Kintz, & Hunter, 2018).This parasite causes Cryptosporidium disease, which results in watery diarrhea and inflammation of the intestines. This parasite can also be transmitted from animals to humans (which is a common disease between humans and animals). The Cryptosporidium parasite spreads all over the world (Baldursson & Karanis, 2011), its transmission related to food (Putignani & Menichella, 2010),, where the parasite Cryptosporidium has been detected in ready-to-eat vegetables and irrigation water in both developed and developing countries (AMORÓS, ALONSO, & CUESTA, 2010).Because it is transmitted through contaminated food, it was described by the World Health Organization as Cryptosporidiosis in 2010 (Kirk et al., 2015), and the rate of outbreaks worldwide reached 239 between 2011 and 2016 (Efstratiou, Ongerth & Karanis, 2017),The World

Health Organization (WHO) also included it in the list of neglected diseases because of the severe symptoms it causes to the host in addition to the economic damages associated with infection (Chalmers & Katzer, 2013).Cryptosporidium is characterized by its wide genetic diversity, which results in the presence of 38 species (Feng, Ryan & Xiao, 2018),Also, its prevalence rate in industrialized countries is 30%, while in developing countries it reaches 90%. Cryptosporidium infection is more prevalent during the rainy and warm months, and Cryptosporidium eggs remain infectious for 6 months (Satoskar et al., 2009).There are several factors that affect the spread of parasites on vegetables and the extent of their spread, which are the treatment procedures for vegetables, climatic conditions, geographical location, types of vegetables, and the quality of irrigation water (Berrouch et al., 2020). Also, the bad health practices during production and transportation carried out by those who deal with vegetables and fruits, including thisConsumers contribute to the contamination of vegetables and fruits (Gupta, Satpati, Nayek, & Garai, 2009) and the chance of vegetable contamination increases when using animal manure that contains viable eggs (Sleman Ali et al., 2018). The morphology of vegetables or fruits has an effect on increasing pollution (Istifanus & Panda, 2018), where the chance of contamination of plants with wrinkled surfaces is more polluted than those with smooth surfaces (Sleman Ali et al., 2018).

Thus, we can describe the contamination of vegetables and fruits by parasitic protozoa that cause diseases such as Cryptosporidium as a global threat to public health, and due to the lack of studies concerned with this aspect in Iraq (Salah al-Din Governorate), as far as we know, this scientific paper was made to assess its spread on vegetables and fruits in sensitive, accurate and simplified ways that can be applied In most laboratories.

2- Materials and methods:

2-1-collect samples:

In this study, (230) samples of fruits and vegetables were collected as follows: 44 samples of lettuce, 32 celery, 30 basil, 35 cress, 21 cucumbers, 29 tomatoes, 15 fruits, apples, and oranges, 24 samples, which were randomly collected from the local markets of the city of Tikrit at a rate of (250 - 1000) g according to the type and were placed in clean plastic bags separately and transferred to the laboratory within less than 24 hours.In the laboratory, each vegetable or fruit sample was washed separately in a container containing 0.1M brine (prepared according to the method (Hernández-Arango et al., 2019). The washing solution was then passed through medical gauze to remove the mud and coarse materials. Then, the washing solution was concentrated The sample was using overnight precipitation and the clear solution that was disposed of was separated and the precipitate was transferred to clean tubes marked according to the type of sample.

2-2 -Microscopic examination:

After the flotation process of the precipitate using a saturated sugar solution, a part of the sample was taken on the slide and left to dry and fixed using absolute ethyl alcohol, then left to dry at room temperature and dyed with carbolfuxine dye and left for 20 minutes, then washed the slide with tap water and added to it sulfuric acid 2% It was washed again with tap water and stained with green malachite dye for 5 minutes and finally washed with water and left to dry at room temperature & Pohlenz, 1981 (Henriksen) and examined under a microscope at a power of 100x, 40x.

2-3 - DNA extraction:

DNA was extracted according to the modified Ali et al., 2008 method, by placing 500 µL of the sample in a 1.5M capacity Abendrov, and adding to it 600 µL of cell contents breaking solution (prepared

according to the method of Ali et al., 2008). The tubes were closed and then mixed using a Vortex apparatus. For one minute until the precipitate is broken down. Then the tubes were placed in a water bath at a temperature of 65 ° C for 60-45 minutes.The tubes were taken out and left to take room temperature. Centrifugation was carried out at 14000 rpm for 2 minutes. Then 500 µL of the upper layer was transferred to a new Eppendorf. 100 µL of NaCl was added at a concentration of 5.3 M and 500 µL of chloroform and . Mix well using a micro pipette and put on a shaker for 10 minutes, then put in the centrifuge for 5

minutes at a speed of 14000 rpm. Transfer 500 µL from the upper aqueous layer to a new Appendorf. 100 µL of ammonia acetate and 500 µL of isopropanol were added to it and left for 10 in the refrigerator at -20 degrees). Then I took out the tubes and turned quietly up and down.Then the tubes were centrifuged at a speed of 14,000 rpm for 5 minutes. The solution was gently disposed of and 500 µL of 70% ethanol was added to purify the precipitate from the previous solutions, then it was placed in a centrifuge also at a speed of 14,000 rpm for 5 minutes. Pour the alcohol gently and then put the tubes in the incubator at 37°C for one hour until the ethanol dries out from the tubes. Then 100 µL of sterile distilled water was added to the DNA precipitated in the Appendorff and for the purpose of dissolving the DNA the tubes were placed in the water bath for 10 minutes at 65°C. The DNA was examined by electrophoresis using 1% agarose gel. Maniatis et al. 2001) as shown in Fig 1.

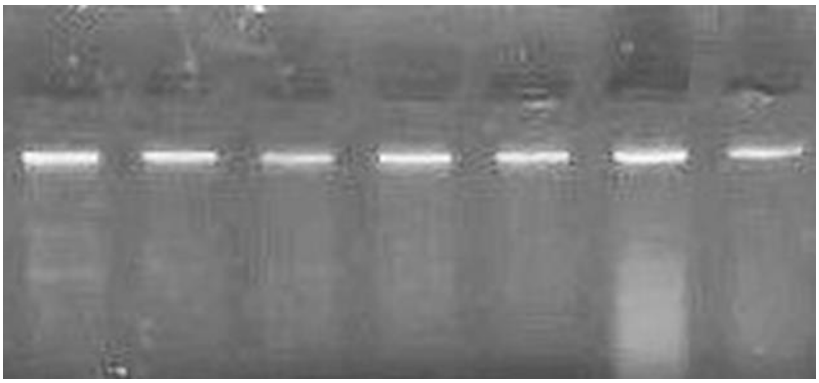

Fig (1) Electrophoresis using 1% agarose gel.

2-4 -DNA sequence analysis:

The HSP-70 gene has been successfully used to diagnose Cryptosporidium in previous epidemiological and taxonomic studies.(Fayer et al: 2005. Langkjær et al; 2007. Mendonc et al; 2007 Castro-Hermida etal ; 2007) each sequence was compared to the GenBank sequence for Cryptosporidium (accession numbers AY151416 and AY741306).

2-5-Amplification of HSP-70 gene fragments of Cryptosporidium by Nested PCR:

Nested PCR was used to amplify the fragments of the HSP-70 gene using the first-round nested PCR protocol.

forward: 50 -GGTGGTGGTACTTTTGATGTATC-30

Reverse : 50 - GCCTGAACCTTTGGAATACG-30

Amplification is a 448-bp packet size (Morgan et al., 2001)). And a 20 µL reaction volume of the following components:

Volume	Component
10 µl	GoTaq®G2Green Master Mix
1 µl	Primer , forward concentration of 10 pmole
1 µl	Primer , reverse concentration of 10 pmole
2 µl	DNA template
µl 6	Nuclease-FreeWater

The components were mixed and the reaction tubes were inserted into the thermal cycler to complete the reaction according to a special program consisting of 35 cycles, each consisting of:start at 94 C for 5 min,94 C for 30 s,58C for 30 ,72 Cfor 30 s,afinal extension step at 72 C for 10 min.As for the second round, the initiator was used:

Forward: 50 -GCTGATGATACTCACTTGGGTGG-30

Reverse :50 - CTCTTGTCCATACCAGCATCC-30

To amplify the 325-bp product, the requirements and reaction conditions were completely identical to those of the first reaction. Fig (2) and (3).

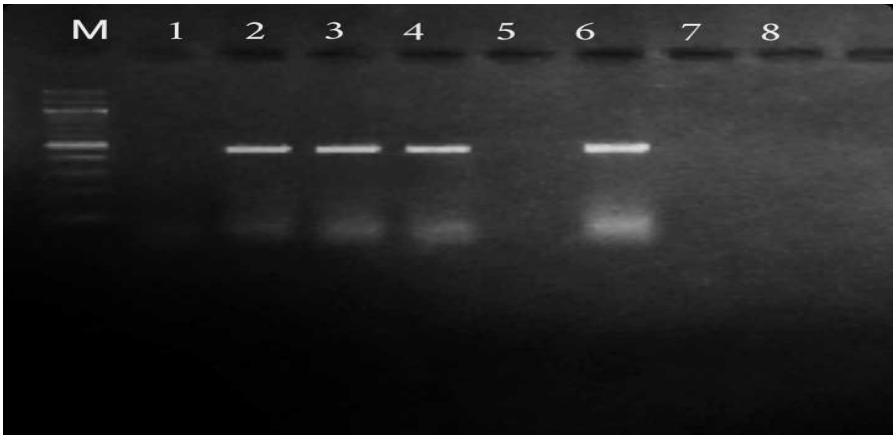

Fig (3). Electrophoresis of the PCR product of the HSP-70 gene on a 2% agarose gel. M: stands for (100bp DNA Ladder). The numbered samples (2,3,4,6) are the positive samples with the appearance of the band448– pb.

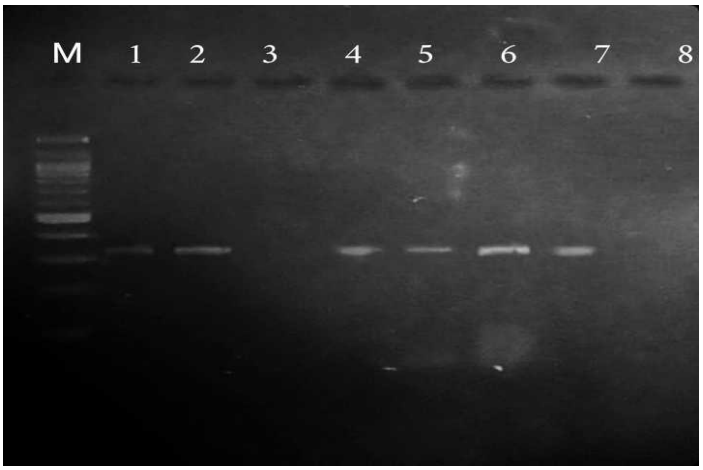

Fig (3). Electrophoresis of the PCR product of the HSP-70 gene on a 2% agarose gel. M: stands for (100bp DNA Ladder). The numbered samples (1,2,4,5,6,7) are the positive samples with the appearance of the band325- pb

3-Results and discussion :

The results of the microscopic examination of the current study showed the presence of the parasite Cryptosporidium spp on vegetables and fruits distributed as in the following table:

Table (1) shows the percentages of Cryptosporidium contamination of vegetables and fruits,

Sample type	Positive sar	Percentage	Negative sar	Percentage	Total	Percentage
Lettuce	2	4.54%	42	%95.46	44	%19
Celery	0	0%	32	100%	32	%13.9
Cress	0	0%	35	%100	35	%15.21
Basil	1	3.33%	29	%96.67	30	%13
tomatoes	0	%0	29	%100	29	%12.6
Cucumber	0	%0	21	%100	21	%9.1
Apple	0	%0	24	%100	24	%10.4
Orange	0	%0	15	%100	15	%6.5
Total	3	%1.30	227	%98.7	230	100%

distributed according to the results of microscopic examination.

The results obtained in the study also showed the possibility of extracting DNA from isolated samples of vegetables and fruits. Where DNA was extracted for 102 vegetable samples out of a total of 230 samples, i.e. 44.34% of the total samples of the study. The reason for the negativity of some samples may be due to the nature of the morphological shape or the fact that their surfaces are smooth and not wrinkled, so the parasite does not stick to them. The results are shown in Table (2).

Table (2) shows the percentages of DNA extraction from samples isolated from vegetables.

Sample type	Positive sa	Percentage	Negative sar	Percentage	Total	Percentage
Lettuce	31	70%	13	30%	44	%19
Celery	27	%84.3	5	15.6%	32	%13.9
Cress	20	%57	15	%46.875	35	%15.21
Basil	24	80%	6	20%	30	%13
tomatoes	0	%0	29	%100	29	%12.6
Cucumber	0	%0	21	%100	21	%9.1
Apple	0	%0	24	%100	24	%10.4
Orange	0	%0	15	%100	15	%6.5
Total	102	%44.3	128	%55.6	230	100%

As for the percentage of the results of the PCR test, it is shown in Table (3). As it was 5 positive samples from 31 samples of DNA isolated from lettuce, i.e. an average of 16% due to the high percentage of this percentage. Several factors, including that Cryptosporidium eggs remain alive on lettuce leaves and thus increase the chance of infection for consumers (Swaffer et al., 2018) or the farms may be irrigated with water contaminated with Cryptosporidium parasite Or that the soil is contaminated with the feces of animals infected with the Cryptosporidium parasite (Xiao et al., 2018)

Two (2) positive samples of 27 DNA were isolated from celery, i.e. an average of 7.40%. We note a decrease in this type of samples. The reason may be the morphology of the celery plant, which does not help in sticking to the eggs Cryptosporidium on it or because of the lack of use of animal fertilizers or the lack of contamination of the soil with the feces of infected animals, as mentioned previously. While the DNA isolated from the cress was not contaminated with Cryptosporidium parasite, this is due to the possibility that the cress during washing releases organic substances such as sugars and resinous substances that hinder the work of the PCR.While 3 samples were positive out of 24 isolated DNA samples from basil, i.e. an average of 12.5%. This result explains the possibility that the morphology of the basil plant helped to stick to Cryptosporidium eggs, or the possibility that the basil was planted in a polluted environment, whether during irrigation or fertilization. In the end, all types of food show characteristics that interfere with the method of extracting protozoa. As the washing solution, the method of washing, the adhesion force shown by the parasite, and the inhibitors present in the sample all interfere with the extraction and affect the PCR result. (Cook et al., 2007).

Table (3) shows the results of the PCR reaction for the HSP-70 gene of the Cryptosporidium parasite.

Sample type	Positive sam	Percentage	Negative sar	Percentage	Total	Percentage
Lettuce	5	%16	26	%84	31	70%
Celery	2	%7.40	25	%92.6	27	%84.3
Cress	0	0%	20	%100	20	%57
Basil	3	%12.5	21	%87.5	24	%80
Total	10	9.80%	92	%91.19	102	%72

At present, PCR technology is used to diagnose parasites in general, as well as parasites isolated from vegetables all over the world, but I rarely use this technology in Iraq and the region for this purpose.

In Brazil, the results of a study converged with the findings of the current study, as it confirmed that the Cryptosporidium parasite prevalence rate on vegetables is 9.5%. (Ferreira et al., 2018). A study in India estimated the rate of contamination of vegetables with the eggs of the parasite Cryptosporidium to be 6% and confirmed that the rate of parasite contamination of lettuce is 0%.

A study in China in Henan Province showed that the prevalence of Cryptosporidium on vegetables and fruits was 3.7% (Li et al., 2019). Another study in Qinghai Province confirmed that the prevalence of the parasite on vegetables and fruits is 2.5% (Li et al., 2020). In South Korea, a study in Seoul found that vegetables sold in local markets were contaminated with Cryptosporidium parasite at a rate of 12.5% (Hong et al., 2014).

In Poland, the prevalence of Cryptosporidium on fresh vegetables was 4.7% (Rzeżutka et al., 2010). We can say that the study achieved its goal through the results that were reached, where the extent of the spread of the parasite Cryptosporidium on vegetables and fruits in the city of Tikrit - Salah al-Din Governorate was determined using light microscopy and overlapping PCR, and the DNA was extracted in a simplified way that can be easily applied.

4- Resources:

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