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# Potential Transmission of Human Fascioliasis Through Traditional Local Foods, in Northern Iran

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## Abstract

Ingestion of infective metacercariae, attached to watercress or other various species of water and terrestrial plants, has been implicated as the main source of human contamination by fasciolid flukes. Presence of several species of aromatic wild grown plants, which are eaten fresh on the table or used for preparation of some plant-made foods (Delar, mixture of salt and ground local plants, as a paste and Zeitoon-Parvardeh , olives in walnut sauce, as an appetizer) have been suggested to play a role in human contamination in the endemic zone of fascioliasis, in Gilan province, northern Iran. The aim of the present study was to evaluate the impact of ingredients using for preparation of these local foods on viability and infectivity of liver fluke metacercariae. Metacercariae for this study were obtained by experimental infections of *Lymnaea gedrosiana*, collected from Bandar Anzali endemic zone. The viability and infectivity of metacercariae kept in Zeitoon-Parvardeh and Delar was checked by microscopical analyses and animal infection assays. The results indicate the possibility of human contamination following consumption of these traditional foods when prepared with fresh vegetables presenting attached metacercariae.

**Keywords:** *Human fascioliasis, Food-borne parasites, Metacercariae, Trematode infection, Iran*

## Introduction

Food-borne trematode infections, including fascioliasis, are among common public health problems worldwide. During the last two decades, the public health importance of human fascioliasis has been increased significantly, with human cases reported from 51 countries in five continents (1). Human infection follows consumption of plants or drinking water contaminated with infective metacercarial cysts of the liver flukes belonging to the genus *Fasciola* (1, 2). Watercress is one of the commonest means of infection, but recent studies have shown that different species of fresh water plants may have a role in various geographical zones (2- 4). In Iran, human fascioliasis was sporadic until 1987 when an outbreak in Gilan province occurred and affected more than 10,000 people (3, 4). A second outbreak occurred some 10 years later, in which once again several thousand people were infected (5). Several hundred cases recorded annually between two epidemics and afterwards, by health centers of Rasht and Bandar-Anzali, indicate the endemicity of the disease and its public health importance in northern Iran (6).

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In the endemic areas of Gilan, there are several kinds of wild grown plants (*Eryngium* spp. and *Mentha* spp.) which are very popular and may be eaten fresh or ground and mixed with walnuts, various spices, garlic and fresh olives for the preparation of an appetizer called Zeitoon-Parvardeh or may be used along with a great quantity of salt for the preparation of a herbal paste called Delar. The amount of salt, which is used for Delar preparation is very high, which is the basis of the local name "green salt". The salt quantity and kind of vegetables in Delar differs somewhat depending on people's tastes in different places. Usually, salt constitutes 30-40% of this paste (w/w). The aromatic vegetables, considered by previous workers as the main source of human infections in this area, are collected by villagers and sold in the streets almost thorough the year (3, 4, 7, 8).

The main objective of the present study was to determine whether the ingredients used in these foods alter the viability and infectivity of the liver fluke metacercariae.

## Materials and Methods

This study was performed in Dept. of Microbiology, Gilan University of Medical Sciences and Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran, with the collaboration of Dept. of Parasitology, University of Valencia, Spain.

***Metacercariae preparation*** Metacercariae were obtained by experimental infections of *L. gedrosiana* (9) collected from their natural environment in Bandar-Anzali water collections. Eggs of *Fasciola gigantica*, which obtained from gallbladder of the cattle, slaughtered in Bandar-Anzali slaughterhouse, were used to prepare miracidia for infecting 150 snails to get metacercariae for studies on mice. In the other hand, eggs obtained from uterus of an adult *F. gigantica*, recovered from the liver of an infected local cattle at Rasht district (capital of Gilan province) were cultured and the miracidia produced, used to infect 120 snails to get metacercariae for studies on hamsters.

The snails were maintained under controlled conditions in the laboratory until the shedding of cercariae. The metacercariae were collected on a plastic sheet and lettuce, and stored at 4°C in natural water for at least two weeks and then added to the foods.

***Delar preparation (mixture of salt and ground local plants)*** *Mentha pulegium* and *Mentha piperita* (locally named Khlivash and Bineh, respectively) were washed and ground thoroughly. Salt was then added to the fresh ground plants and mixed well (40% on the basis of weight). The pH, measured with an electrical pH-meter (CRISON micro pH 2000), was 5.0.

***Zeitoon-Parvardeh preparation (olives in walnut sauce)*** Sufficient amounts of *Eryngium coucasicum* (Choochagh), walnuts and garlic were ground. The stones of olives were removed and the ground materials thoroughly mixed with olives. Spices and sour-pomegranate juice were added afterwards.

***Collection and inoculation of metacercariae*** The metacercariae obtained through experimental infections were divided into 3 groups; some were added to Delar, some kept in Zeitoon-Parvardeh and some were stocked in water as controls. During the process of food preparation by a native person, metacercariae were added, mixed thoroughly and kept at 4 °C until animal inoculation. To recover sufficient number of metacercaiae, a dilute suspension of the foods in water was prepared in a petridish so that under a stereomicroscope the metacercariae could be found and collected. The metacercariae were then inoculated orally to the mice and hamsters.

**Verification of metacercarial viability**

***Metacercarial viabitlity checked by microscopical analysis*** The viability of metacercariae in Delar and Zeitoon-Parvardeh was checked weekly according to the refractile appearance of secretory granules in the cysts (10). Metacercarial cysts recovered from the foods

were studied between glass slide and coverslip under a light microscope. This method was used for Zeitoon-Parvardeh just for two weeks after preparation. This food is served as an appetizer along with the main dish in endemic areas and may be eaten right after preparation and not stored more than two weeks, as it is usually spoiled by molds. For Delar, the verification of metacercarial viability was continued up to four weeks. Although Delar is consumed immediately after preparation but it may be stored and utilized for more than six months. It is usually eaten with cucumber, green prune, yoghourt etc.

***Metacercarial viability checked by animal infection*** All animals were orally infected with metacercariae. The infected animals were kept in an animal house under daily observation. Finally, the animals were euthanized, and their liver, abdominal subcutaneous, peritoneal and thoracic cavities checked for adult flukes using a stereomicroscope (11).

***Experiment I: Mice (Swiss)*** Thirty female Swiss mice were divided into two identical groups (15 mice/group). Each mouse was orally inoculated with 10 metacercariae. In group I, the metacercariae used were those that had been kept in Delar for 30 d, and in group II (control group) metacercariae had been kept for the same time at 4°C in water. Ten weeks post-infection all mice in the test and control groups were euthanized, and checked for the presence of adult flukes and pathological lesions.

***Experiment 2: Hamsters*** Twenty one golden hamsters were divided into three groups (7 hamsters/ group). Each hamster was orally inoculated with 25 metacercariae. Hamsters in group III, IV and V received metacercariae kept in Delar, Zeitoon-Parvardeh and water for 7 d, respectively. Eight weeks post-inoculation all hamsters in the test and control groups were studied. Non-parametric test (Mac Nemar) was used for statistical analysis.

## Results

***Metacercarial viability checked by microscopical analysis*** The results of the metacercarial viability, obtained by microscopical examination, are shown in Table 1. About 65% of the cysts collected from Zeitoon-Parvardeh and 58% from Delar, were still alive two weeks after food preparation. In experiment I, the percentage of viable metacercariae in Delar, Zeitoon-Parvardeh and water in days 1, 7 and 14 were not significantly different but when the number of viable metacercariae in Delar and water at days 21 and 28 were compared, a significant difference was found ($P$= 0.0171 and $P$= 0.003, respectively).

**Metacercarial viability checked by animal infection**

***Mice*** The results of the experimental infection in mice are shown in Table 2. Two out of 15 mice in group I (13.3%) were infected, and one fluke was recovered from each. Two apparently non-infected mice showed some pathological changes, but no flukes were recovered. In group II, three mice (20%) were infected, from which one mouse had 3 flukes but each of the other two, harbored only one. All these flukes morphologically resembled *F. gigantica* and were recovered from different parts of the body, as seen in Table 2. The percentages of worms recovered (number of total flukes recovered / number of total metacercariae inoculated X 100) in groups I and II were 1.33 and 3.33, respectively. One mouse in the control group showed some pathological changes in the liver but no flukes were recovered. The number of infected animals and parasites recovered in this study (Table 2) was higher in the control group receiving untreated cysts.

***Hamsters*** The results of the experimental infection in hamsters are shown in Table 2. In all groups, the viability of metacercariae is verified. The infectivity percentages of animals in groups III (Delar), IV (Zeitoon-Parvardeh) and V (water) were 28.57%, 71.4% and 57.14%, respectively. The percentage of worms recov-

ered in hamsters was 2.3%, 3.4% and 4.6% in the same groups. The percentage of worms recovered in control group (group V) was higher than both test groups (groups III and IV). This difference was more prominent between control and Delar groups. In all infected animals, the evidence of mild to severe pathology (mostly severe) on the surface and in parenchyma of the liver was evident. One hamster in control group and one in Delar group showed severe pathology, both on the surface and in parenchyma of the liver, but no flukes recovered.

**Table 1:** Viability of *Fasciola gigantica* metacercariae checked by microscopical analysis according to the refractile appearance of secretory granules and movements of the juvenile within the cyst

| **Experiment** | **1** | | | **2** | | |
|---|---|---|---|---|---|---|
| **Medium** | **Delar** | **Zeitoon-Parvardeh** | **Water** | **Delar** | **Zeitoon-Parvardeh** | **Water** |
| **Nº metacercaria Days** | **a/ch* (%)** | **a/ch (%)** | **a/ch (%)** | **a/ch (%)** | **a/ch (%)** | **a/ch (%)** |
| 1 | 23/23 (100) | 14/15 (93.3) | 15/15 (100) | - | - | - |
| 7 | 13/17 (76.5) | 17/21 (80.9) | 15/15 (100) | 19/21 (90.5) | 17/18 (94.4) | 13/13 (100) |
| 14 | 11/19 (57.8) | 8/12 (66.6) | 14/15 (93.3) | - | - | - |
| 21 | 14/23 (60.8) | - | 15/15 (100) | - | - | - |
| 28 | 8/17 (47) | - | 15/15 (100) | - | - | - |

* = a (number of alive metacercariae)/ ch (number of checked metacercariae). a/ch = frequency.

**Table 2:** Results obtained in experimental infections of mice and hamsters with metacercariae of *Fasciola gigantica* (cattle isolate, Gilan, Iran) treated in Delar and Zeitoon-Parvardeh.

| | **Groups of animals** | | | | |
|---|---|---|---|---|---|
| | **Mice** | | **Hamsters** | | |
| | **I Delar** | **II Water** | **III Delar** | **IV Z-P●** | **V Water** |
| Nº animals exposed | 15 | 15 | 7 | 7 | 7 |
| Nº cysts inoculated | 10 | 10 | 25 | 25 | 25 |
| Nº animals infected | 2 | 3 | 2 | 5 | 4 |
| Nº flukes recovered | 2 | 5 | 4 | 6 | 8 |
| % animals infected | 13.3 | 20 | 28.6 | 71.4 | 57.1 |
| % flukes recovered■ | 1.33 | 3.33 | 2.28 | 3.42 | 4.57 |
| Nº deaths before necropsy | 2 (0,2)* | 1 (0,1)* | 2 (1,1)* | 2(2,0)* | 4 (3,1)* |
| Liver microhabitat | - | 2 | 4 | 5 | 4 |
| Peritoneum microhabitat | 1 | 1 | - | 1 | 4 |
| Subcutaneous microhabitat | 1 | 2 | - | - | - |
| Liver pathology present | 2 | 3 | 3 | 6 | 5 |
| Liver pathology absent | 13 | 12 | 4 | 1 | 2 |
| Metacercaria in each medium (days) | 30 | 30 | 7 | 7 | 7 |

● = Zeitoon-Parvardeh

* = n (p+, p-) n= number of animals died before necropsy, (P+ = parasite positive, P-= parasite negative).

■ = (number of total flukes recovered / number of total metacercariae inoculated to all animals in related group X 100)

## Discussion

Human fascioliasis is endemic in many countries of the world, where people eat fresh aquatic vegetables on which infective metacercarial cysts of the liver fluke have been encysted (1, 4). Studies carried out in human hyper endemic areas, such as Bolivia (12) and Egypt (13, 14), have shown that many raw-eaten, cultured or wild-grown plant species are involved in human contamination. Consumption of aromatic plants such as *Eryngium* spp. and *Mentha* spp., whether eaten fresh or prepared in some popular kinds of pastes or appetizers, has been mentioned as the most probable source of human infections in the endemic areas of Gilan (3, 4, 7, 8). Although in Iran, fascioliasis is prevalent in livestock throughout the country, human infection with fasciolids is mainly reported in this littoral region of the Caspian Sea (15). Since 1998**,** there have been two large outbreaks of human fascioliasis in Gilan Province in which more than 15,000 people were infected (3-6). Nevertheless, sporadic cases of the disease have already been reported from different parts of Iran, as well as northern provinces of Gilan and Mazandaran (16-18).

In spite of high prevalence of food borne trematode infections, the number of studies analyzing the effect of various foods processing on the viability and infectivity of the metacercariae is very small. The effect of acidity and salt appears to be studied only on trematode species whose metacercariae encysted in a second intermediate host, for instance fish. In *Opisthorchis* species and the heterophyid *Haplorchis taichui*, the viability of metacercariae is affected by salt, acidity or fermentation in a period between 3 h and 24 h (19-21). *Clonorchis sinensis* is the only species whose metacercarial viability does not seem to be affected, at least in 30% salt concentration throughout a period of 5-7 d (22).

This research appears to be the first to study the effects of traditional food preparation and conservation on the metacercariae of liver fluke species of the genus *Fasciola.* Microscopical observation (Table 1) showed that, up to two wk after food preparation the viability of liver fluke metacercariae kept in both Zeitoon-Parvardeh and Delar appears to be unaffected or only slightly decreased. Approximately half of the metacercariae remained viable, 3-4 wk after the preparation of Delar, indicating a high infection potential.

Experimental animal infection tests proved that metacercariae might keep their infectivity in both types of traditional local foods (Table 2). Experiments on mice showed that the number of both infected animals and flukes recovered, were higher in the control group (metacercariae kept in water) than in the Delar group, suggesting that the high salt concentration used for Delar preparation may alter the infectivity of the metacercariae.Comparing the study on mice with that on hamsters, a lower infection rate and a higher ectopic location of the flukes in mice indicates that, this animal is not a suitable host for *F. gigantica*. This finding is in agreement with previous reports (23). In the other hand, as hamster was found to be an appropriate host for *F. gigantica,* therefore the results obtained for the studies on this animal is more conclusive. The number of hamsters infected by metacercariae kept in Zeitoon-Parvardeh was higher than that infected by metacercariae from Delar, and metacercariae kept in Zeitoon-Parvardeh and Delar yielded less flukes than those stored in water. This indicated that, the ingredients of these foods have some negative impact on metacercarial infectivity. Furthermore, Delar appeared to be more detrimental perhaps due to high concentration of salt.

These findings suggest that in both foods, metacercarial infectivity is decreased when compared to the control group, but still the metacercariae may remain infective. It is also clear that viability and infectivity are both reduced with time. This may also be a reflection of the aging of the metacercariae, which was previously reported to have negative effects on both viability

and infectivity of the cysts (11). This study indicates that most metacercariae keep their viability and infectivity even several weeks after food preparation, and the traditional methods of preparing Zeitoon-parvardeh and Delar (salt= 40%; pH= 5) have little effect, if any, on viability and infectivity of the metacercariae, however, the high concentration of the salt in Delar may be more detrimental in time.

Therefore we consider both Zeitoon-parvardeh and Delar, as potentially hazardous when prepared with fresh local vegetables, if contaminated.

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