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PLANT-BORNE HUMAN CONTAMINATION BY FASCIOLIASIS

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Abstract. Contamination by fasciolids takes place through ingestion of metacercariae attached to vegetables. Experimental studies were performed with plant-made foods suggesting a role in human contamination in Iran and on the usefulness of potassium permanganate as a preventive tool for killing metacercariae attached to vegetables used in salads in Egypt. In the foods assayed, although viability decreases with time, a high percentage of the metacercariae were still alive 2 and 4 weeks after preparation. Infection of laboratory animals proved that metacercariae kept their infectivity. The 5-minute tests of potassium permanganate effects showed that metacercarial viability was not affected even at the very high doses of 300, 600, and 1,200 mg/L. Careful, subsequent washing of leaves and vegetables with water is therefore needed after its application. A review on similar studies performed with metacercariae belonging to fasciolid and other trematode species affecting humans is included.

INTRODUCTION

The need to implement control measures against important food-borne trematode infections has recently been emphasized.[1] Among them, plantborne trematodiases have been included in the list considered by the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition.[2] Fascioliasis and other food-borne trematodiases were added to the list of important helminthiases with a great impact on human development at the Third Global Meeting of the Partners for Parasite Control held in World Health Organization (WHO) Headquarters in Geneva in November 2004.

Fascioliasis is caused by digenean species of the genus *Fasciola* (Fasciolidae): *F. hepatica* in Europe, Asia, Africa, the Americas, and Oceania; *F. gigantica* in Asia and Africa. A different fasciolid species belonging to another genus, *Fasciolopsis buski*, causes fasciolopsiasis in Asia. These three species are transmitted by freshwater snail vectors. Rediae produce cercariae that emerge and swim in water until encystment on a substratum, mainly aquatic plants. Human contamination takes place through ingestion of infective metacercariae.[3]

Concerning fascioliasis, recent studies have shown that there are more contamination sources than the one traditionally noted, through free-living (= non-parasitic), encysted metacercariae attached to watercress.[4] The most important human infection sources seem to be 1) ingestion of wild freshwater plants; 2) ingestion of cultivated freshwater plants; 3) ingestion of wild terrestrial plants; 4) ingestion of cultivated terrestrial plants; and 5) drinking of beverages made from local plants.[5]

Consequently, studies on the viability and infectivity of metacercariae and on strategies and agents potentially useful as control measures to impede human contamination by metacercariae-carrying vegetables and plant-made foods become crucial. Different studies have furnished a baseline on metacercarial viability and infectivity.[6,7] Metacercarial infectivity is dependent on storage time, being lower when metacercariae are older. Moreover, metacercarial viability and infectivity did not show differences between isolates from different reservoir species.[7,8]

However, the number of studies on strategies and agents potentially useful as control measures to impede human contamination is surprisingly low. The purpose of this paper is to contribute to filling this gap by exposing results obtained in experimental studies performed with 1) local Iranian, plant-made foods served as an appetizer or a paste that have been suggested to play a role in human contamination in the northwestern endemic province of Gilan, at the Caspian Sea, where human fascioliasis is a public health problem, including outbreaks involving thousands of individuals[1,9–11] and 2) on the potential usefulness of potassium permanganate, which has been suggested to be the most effective preventive tool for killing metacercariae attached to leaves and vegetables used in salads.[12,13]

Until a few years ago, the use of agents useful to wash off the metacercariae was neglected mainly because people living in poor, rural endemic areas were not expected to use them. However, today, fascioliasis is an emerging/re-emerging disease in many countries; infected patients are increasingly being diagnosed in large urban areas such as La Paz and El Alto (Bolivia), Alexandria, Damanhour and Cairo (Egypt), and Bandar Anzali and Rasht (Iran).[5,10,11,14] In these cities, agents such as potassium permanganate are easily available and could thus be very useful for individual prevention if they are effective.

A short review on similar studies performed with metacercariae belonging to fasciolid and other trematode species affecting humans is also included.

MATERIALS AND METHODS

Experiments with Iranian local foods. *Gilan traditional foods assayed.* In the Iranian province of Gilan, there are several very popular kinds of wild plants, such as species of *Eryngium* and *Mentha*, which are eaten raw, ground and mixed with walnuts, various spices, garlic, and fresh olives for the preparation of an appetizer called "zeitoon-parvardeh," or used in the preparation of a paste called "delar" along with

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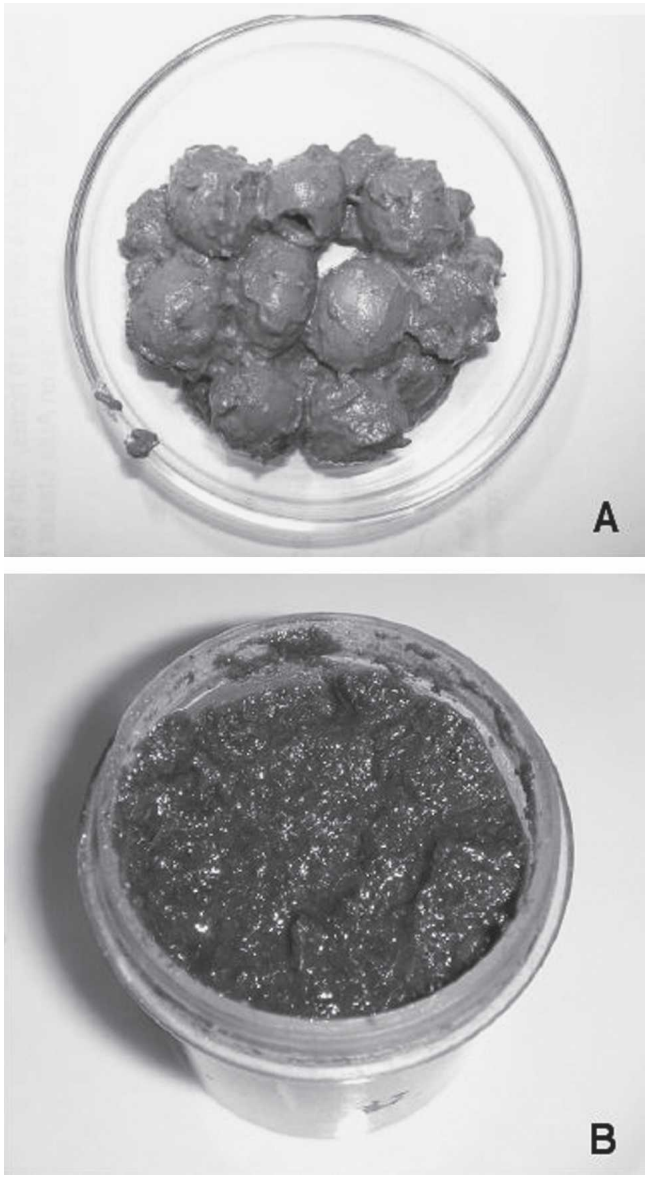

FIGURE 1. Traditional culinary specialties made from very popular kinds of aromatic wild plants, involved in human contamination by fasciolid liver flukes in the endemic province of Gilan, Iran. (**A**) Zeitoon-parvardeh, an appetizer dish, which is made by mixing the grounded local wild plants with other ingredients and fresh olives. (**B**) Delar, a traditional herbal paste, which may be stored for consumption over several months.

a great quantity of salt. The very high quantity of salt used for delar preparation is at the base for the local name "green salt" also given to this specialty. The amount of salt and kinds of vegetables in delar differ somewhat depending on geographical zones and people's habits (usually 30–40%). However, in some regions, the mean weight of salt is almost equal to that of vegetables used (50%). The aromatic vegetables used for these two traditional foods are usually sold throughout the year, mainly in the streets of all endemic areas in Gilan Province. Although the consumption of these plants, raw or elaborated in those appetizer and paste, has been suggested to be the main source of human infections, no study on the viability of liver fluke metacercariae in zeitoon-parvardeh and delar under typical preparation conditions has been performed thus far.

For zeitoon-parvardeh preparation (Figure 1A), plants of the species *Eryngium coucasicum* (locally known as "choochagh"), walnuts, and garlic were ground. Ground materials were thoroughly mixed with olives after the removal of olive stones. Spices and sour-pomegranate juice were added afterward. This foodstuff is served as an appetizer and may be eaten right after preparation. However, it cannot be stored for > 2 weeks because growth of fungi prevents its further consumption.

For delar preparation (Figure 1B), amounts of *Mentha pulegium* and *Mentha piperita* ("khlivash" and "bineh," respectively) were washed and ground thoroughly using an electrical grinder or an ancient instrument (locally named "namakyar"). After thorough grinding, salt (40%) was added to the raw plants and mixed well. Delar is edible after preparation, but the paste may be stored for consumption over several months. It is usually eaten with cucumber, prunes, yogurt, etc. The pH, measured with an electrical pH meter (Crison Micro pH 2000, Barcelona, Spain), was 5.0.

Liver fluke metacercariae. To determine whether the ingredients used in the appetizer or paste alter the viability of liver fluke metacercariae, the following two experiments, using snails of the species *Lymnaea gedrosiana*[15] collected from water bodies around the locality of Bandar Anzali, were carried out. 1) Miracidia from fasciolid eggs collected in bile from gallbladders of cattle naturally infected with, the most prevalent fasciolid species,[16] from the Bandar Anzali slaughterhouse, were used to infect 150 snails; metacercariae obtained from cercariae shed by the snails were used to orally infect mice. 2) Miracidia from eggs obtained from the uterus of *F. gigantica* adults recovered from livers of naturally infected cattle slaughtered in Rasht, capital of Gilan Province, were used to infect 120 snails; metacercariae obtained from cercariae shed by the snails were used to orally infect hamsters. Metacercariae were collected on a plastic sheet and lettuce and stored in drinking water at 4°C for 2 weeks before adding them to the prepared foods.

Metacercariae were divided into three groups: 1) added to zeitoon-parvardeh; 2) added to delar; and 3) kept in water as control. During the process of food preparation by a native person, metacercariae were added and mixed thoroughly. Finally, the foods were kept at 4°C until oral infection of mice and hamsters. For animal infection, metacercariae were collected, by means of a pipette, from a small amount of zeitoon-parvardeh and delar previously put in a Petri dish containing water.

Verification of metacercarial viability and infectivity. The viability and infectivity of metacercariae kept in zeitoon-parvardeh and delar was checked by microscopical analyses and animal infection assays performed in a laboratory of the Gilan University of Medical Sciences.

Microscopical analyses included the weekly checking of metacercarial viability according to the refractile appearance of secretory granules.[17] Metacercarial cysts recovered from the foods were studied between a glass slide and coverslip under a light microscope. This method was used for metacercariae kept in zeitoon-parvardeh only for 2 weeks after food preparation and before contamination with fungi. For delar, the verification of metacercarial viability was continued throughout a longer period of 4 weeks after preparation, just until infection of mice, as this culinary speciality can be stored for use over several months.

For animal infection analyses, mice and hamsters were kept

in an animal house under daily observation until killed. The study was approved by the institutional committee on animal care of Gilan University of Medical Sciences (Iran). Their liver, abdominal subcutaneous, peritoneal, and thoracic cavities were checked under a stereomicroscope for flukes.[7] Animals were used in the above-mentioned experiments as follows. 1) A total of 30 female Swiss mice were divided into two identical groups (15 mice/group); each mouse was orally infected with 10 metacercariae; in group 1, metacercariae were kept in delar for 30 days, and in group 2, metacercariae were kept for the same time in water at 4°C as controls. All mice in the test and control groups were analyzed at 10 weeks postinfection (pi). 2) A total of 21 golden hamsters were divided into three groups (7 hamsters/group); each hamster was orally inoculated with 25 metacercariae. In group 3, metacercariae were kept in delar for 7 days, in group 4, metacercariae were in zeitoon-parvardeh for 7 days, and in group 5, metacercariae were kept in water at 4°C for 7 days as a control. All hamsters in the test and control groups were analyzed at 8 weeks pi.

Experiments with potassium permanganate. *Liver fluke metacercariae.* Metacercariae were obtained after cercarial shedding obtained in a laboratory of Valencia University from two naturally infected lymnaeid snails of the species *Galba truncatula* collected in the human hyperendemic area of the governorate of Behera, Nile Delta region, Egypt,[18] and kept in drinking water in a Petri dish under conditions of a temperature of 20°C, photoperiod of 12-hour/12-hour light/dark, and relative humidity of 90% in a climatic chamber Heraeus-Vötsch HPS-500 (Heraeus-Vötsch GmbH, Balingen, Germany). The classification of the metacercariae as belonging to *F. hepatica* was verified by obtaining the adult stage and eggs in laboratory Wistar rats (Iffa Credo, Barcelona, Spain) after experimental oral inoculation by means of a gastric tube. The animals were housed in Micro-Isolator boxes (Iffa Credo, Barcelona, Spain) and maintained in a pathogen-free room, electrically heated with a 12-hour/12-hour light/dark cycle (conditions in compliance with the European Agreement of Strasbourg, 18 March 1986). Food and water were provided *ad libitum*.[7]

Potassium permanganate application. Potassium permanganate is a water-soluble solid, very dark purple, crystalline product, used chiefly as an oxidizing agent, disinfectant, and in medicine as an astringent and antiseptic. A total of 87 metacercariae encysted on lettuce were collected. The metacercariae were stored in drinking water in total darkness at 4°C until required. Only metacercariae of the same age of 15–30 days were used for the test. The effect of potassium permanganate ($KMnO_4$) on metacercariae was tested for a period of 5 minutes at laboratory temperature (22°C) by submerging metacercariae in different water concentrations of 300, 600, and 1,200 mg/L. The experiment was carried out twice, including controls (metacercariae not treated with potassium permanganate) also in duplicate (Table 3). After exposure, metacercarial cysts were washed with modified Earle saline medium.[19]

Verification of metacercarial viability. The viability of metacercariae was analyzed by their *in vitro* excystation capability.[20] Metacercariae were placed in a Petri dish with natural water, and the outer cyst walls were removed by gently pressing with a dissecting needle under a stereomicroscope. Once the outer wall was removed, metacercariae were transferred to a test tube with 10 mL of modified Earle saline medium[19,21] containing 39 mmol/L $NaHCO_3$, 103 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L NaH_2PO_4, 0.8 mmol/L $MgSO_4$, 1.8 mmol/L $CaCl_2$, and 11 mmol/L glucose. Metacercariae were activated by incubation for 1 hour at 38°C in this medium in a shaking water bath (100 strokes/min) under a constantly refreshed 60% CO_2/40% N_2 gas phase. During this experimental phase, a pH of 6.3 was maintained. The incubation was continued for another 30 minutes in aerobiosis, during which the gas mixture was removed and air was used to restore the initial pH of 7.2. After this activation phase, the total volume was transferred to a Petri dish for observation and counting of the activated metacercarial number. Metacercariae were considered activated when presenting movements. Afterward, the medium was returned to the crystal test tube. To stimulate the excystment, taurocholic acid (9 g/L) was added to the medium, and metacercariae were incubated in aerobiosis for another 6 hours at the same temperature (+38°C) and shaking speed (100 strokes/min). After incubation, the total volume of the medium with metacercariae was transferred to a Petri dish and examined under a black-bottomed stereomicroscope. The number of empty cyst walls, free mobile juvenile flukes, and unexcysted metacercariae were surveyed, and the percentage of excystation was calculated as the number of empty cyst walls (verified by observation of mobile free juvenile flukes) from the total number of initially incubated metacercariae. All excystment assays were made in duplicate, and data are expressed as the mean of the two observations.

Statistical analyses. The *n*/*tn*% (n = number of metacercariae found alive or empty cyst walls; tn = total number of metacercariae microscopically checked), with its 95% confidence interval (95% CI; EPIINFO), was used for the statistical analysis of the viability of metacercariae. Because the size of the samples was small (results obtained were so clear that no additional assays were needed), it was considered that significant differences ($P < 0.05$) in *n*/*tn*% were present when there was no overlapping in the values of 95% CIs.

RESULTS

Metacercariae kept in traditional foods. Results of microscope analyses to check metacercarial viability are shown in Table 1. In both zeitoon-parvardeh and delar, although viability seems to decrease with time, a total of 66.6% and 57.8% of the metacercariae were still alive 2 weeks after food preparation, respectively. The statistical comparison of the percentages of living metacercariae in relation to the total number of metacercariae checked in experiment A does not show any significant difference between viability of metacercariae kept in zeitoon-parvardeh, delar, and water (= control) at days 1, 7, and 14.

In delar, metacercarial viability is lower than in water in the third and fourth weeks, differences being statistically significant. However, although delar seems to decrease the viability of metacercariae with time, there is still a relatively high proportion of metacercariae surviving at days 21 and 28 (60.8% and 47.0%, respectively).

Results of experimental animal infection tests to check metacercarial infectivity are shown in Table 2. Among the 15 mice experimentally exposed to metacercariae from delar and dissected at 10 weeks pi, 2 mice (13.3%) were infected with one fluke each and another 2 showed pathologic effects, al-

TABLE 1

Viability of *F. gigantica* metacercariae kept in traditional Iranian foods (zeitoon-parvardeh and delar) and water (= control), checked by microscopical analysis according to the refractile appearance of secretory granules and movements of juveniles within cysts

	Experiment A			Experiment B		
Days	Zeitoon-parvardeh [*n/tn* (%)]	Delar [*n/tn* (%)]	Water [*n/tn* (%)]	Zeitoon-parvardeh [*n/tn* (%)]	Delar [*n/tn* (%)]	Water [*n/tn* (%)]
1	14/15 (93.3)	23/23 (100)	15/15 (100)			
	71.26–99.66	87.78–100	81.89–100			
7	17/21 (80.9)	13/17 (76.5)	15/15 (100)	17/18 (94.4)	19/21 (90.5)	13/13 (100)
	60.19–93.63	52.50–92.04	81.89–100	75.51–99.72	71.94–98.37	70.41–100
14	8/12 (66.6)	11/19 (57.8)	14/15 (93.3)			
	37.68–88.39	35.36–78.17	71.26–99.66			
21		14/23 (60.8)	15/15 (100)			
		40.20–78.24	81.89–100			
28		8/17 (47.0)	15/15 (100)			
		24.78–70.26	81.89–100			

Ranges are 95% CI.

Experiment A, metacercariae obtained from cercariae shed by *L. gedrosiana* snails experimentally infected by miracidia from fasciolid eggs collected in bilis from gallbladders of naturally-infected cattle from Bandar Anzali slaughterhouse; Experiment B, metacercariae obtained from cercariae shed by *L. gedrosiana* snails experimentally infected by miracidia from eggs obtained from the uterus of liver fluke adults recovered from livers of naturally infected cattle from Rasht slaughterhouse; *n,* number of metacercariae found alive; *tn,* total number of metacercariae microscopically checked.

though no fluke was recovered. Among the 15 mice infected with control metacercariae (from water), 3 mice (20.0%) were infected by one, one, and three worms each. The percentage of fluke recovery was lower with metacercariae from delar than with those from water (1.3 and 3.3, respectively). Flukes were recovered from different body parts (Table 2), and one mouse in the control group showed liver pathology but no fluke.

The results of the experimental infection of hamsters showed infectivity percentages of 42.8%, 71.4%, and 57.1% with metacercariae kept in delar, zeitoon-parvardeh, and water (= control), respectively. Percentages of flukes recovered were 2.3%, 3.4%, and 4.6% with the same metacercarial groups, respectively. In all infected hamsters, mild to mostly well-patent pathology on the surface and in the parenchyma of the liver was present. There was severe pathology in one hamster of the delar group and another of the control water group, both in the liver surface and parenchyma, although no flukes were recovered.

Metacercariae treated with potassium permanganate. Results of the tests of potassium permanganate effects on the viability of the metacercariae are shown in Table 3. The results obtained show that metacercarial viability is not affected by potassium permanganate, even at the very high doses of 300, 600, and 1,200 mg/L. The analyses of *n*/*tn*% of control metacercariae in relation to metacercariae treated with the different concentrations showed no significant difference ($P < 0.005$) between them in any group.

DISCUSSION

Effects of food handling and control measures against trematode metacercariae. Despite human infection by foodborne trematodes being very common in numerous countries,[1,3,4] the number of studies on food handling effects (elaboration and conservation conditions) and agents potentially useful as control measures to impede human contamination is relatively small. A summarized review of studies performed from these points of view on metacercariae belonging to fasciolid and other trematode species affecting humans is included in Table 4.

The effect of acidity and salt has been analyzed only in trematode species whose metacercariae encyst in a second intermediate host (i.e., fish). In *Opisthorchis* species and the heterophyid *Haplorchis taichui*, the viability of metacercariae is affected by salt, acidity, and fermentation in a period ranging from 3 to 24 hours.[22–24] *Clonorchis sinensis* is the only species whose metacercarial viability does not seem to be affected, at least in a 30% salt concentration throughout a period of 5–7 days.[25]

TABLE 2

Viability of *F. gigantica* metacercariae kept in traditional Iranian foods (zeitoon-parvardeh and delar) and water (= control), checked by experimental oral infection in laboratory animals

	Mice (experiment A)		Hamsters (experiment B)		
	Delar	Water	Delar	Zeitoon-parvardeh	Water
Days metacercariae in each medium	30	30	7	7	7
No. metacercariae inoculated per animal	10	10	25	25	25
No. animals exposed/infected at dissection*	15/2	15/3	7/2	7/3	7/1
No. animals died before dissection time (+/–)†	2 (0/2)	1 (0/1)	2 (1/1)	2 (2/0)	4 (3/1)
No./percentage flukes recovered per animal	2/1.33	5/3.33	4/2.28	6/3.42	8/4.57
No. animals with flukes in liver/peritoneum/subcutaneous location	–/1/1	2/1/2	4/–/–	5/1/–	4/4/–
No. animals with/without liver pathology	2/13	3/12	3/4	6/1	5/2

Experiment A, mice infected with metacercariae obtained from cercariae shed by *L. gedrosiana* snails experimentally infected by miracidia from fasciolid eggs collected in bilis from gallbladders of naturally infected cattle from Bandar Anzali slaughterhouse; experiment B, hamsters infected with metacercariae obtained from cercariae shed by *L. gedrosiana* snails experimentally infected by miracidia from eggs obtained from the uterus of liver fluke adults recovered from livers of naturally-infected cattle from Rasht slaughterhouse.

* Dissection of mice at 10 weeks post-infection (pi) and of hamsters at 8 weeks pi.

† Animals died before dissection time bearing (+) and non–bearing (–) flukes, respectively.

TABLE 3
Test results of potassium permanganate effects on the viability of *F. hepatica* metacercariae

	Doses of potassium permanganate							
	No of metacercariae used for the test at different doses (mg/L)				Results of viability of test			
	0*	300	600	1,200	No. empty metacercarial cysts	No. excysted larvae	Percent effectivity excystment	95% CI
First tests	7	–	–	–	4	4	57.14	20.23–88.19
	10	–	–	–	4	3	40.00	13.69–72.63
	–	10	–	–	2	1	20.00	3.54–55.78
	–	–	10	–	2	2	20.00	3.54–55.78
	–	–	–	10	2	2	20.00	3.54–55.78
Second tests	10	–	–	–	3	1	30.00	8.09–64.63
	–	10	–	–	0	0	0	0
	–	–	10	–	3	3	30.00	8.09–64.63
	–	–	–	10	3	3	30.00	8.09–64.63
Average	27	–	–	–	11	8	40.74	23.01–60.59
	–	20	–	–	2	1	10.00	1.75–33.12
	–	–	20	–	5	5	25.00	9.59–49.41
	–	–	–	20	5	5	25.00	9.59–49.41

* Controls.

Concerning human fascioliasis, studies carried out in human hyperendemic areas have shown that many raw-eaten, cultured, or wild-grown plant species are involved in human contamination, for example, in Bolivia[26] and Egypt.[12,27] Human contamination by fascioliasis through ingestion of metacercariae included in local beverages is also known in other human endemic areas, as Cape Verde.[1,5] However, this study seems to be the first to analyze the effects of traditional food preparation and conservation on the metacercariae of liver fluke species of the genus *Fasciola*.

This is, nevertheless, not the first study of this kind on free-living metacercariae encysted in the external environment (i.e., non-parasitic in an intermediate host). Several analyses were carried out on metacercariae of the Giant Asian intestinal fluke *F. buski* a long time ago (Table 4).[3] Metacercariae of *F. buski* were killed by 1.0% hydrochloric acid in 18 days, by 2% acetic acid in 9 days, by 3.0% acetic acid in 6 days, by 5% salt solution in 3 hours, by soybean sauce in 30 minutes, by 10% cane sugar in 3 days,[28] by direct solar radiation in 20–30 minutes,[29] by desiccation in 19 hours at 27°C, and by boiling water in 1–2 minutes.[30]

Articles on the efficacy of different agents on *Fasciola* metacercariae are restricted to only two studies in Egypt.[12,27] These studies concluded that the fasciolid metacercarial detaching capacity of running water was very low compared with the high capacity of citric acid (10 mL/L), commercial vinegar (120 mL/L), liquid soap (12 mL/L), potassium permanganate (24 mg/L),[12] and sodium dichloroisocyanurate (1 mg/L).[27] The latter two are, moreover, considered the only ones able to kill the metacercariae.[12,27] The commercial availability of potassium permanganate makes it the agent of choice.

Metacercariae kept in traditional foods. Microscopical analyses (Table 1) showed that, during the 2 weeks between food preparation and consumption, the viability of liver fluke metacercariae kept in both zeitoon-parvardeh and delar is unaffected or only slightly decreased. During the third and fourth weeks in delar, around one half of the metacercariae kept their viability, indicating a high infection potential is still present despite having been in the food for a long time.

Experimental animal infection tests proved that metacercariae might keep their infectivity in both traditional Iranian foods (Table 2). Experiments performed with mice showed that the number of both infected animals and flukes recovered was 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 metacercariae present. However, the host microhabitats other than liver where flukes were found (subcutaneous tissues of the abdominal region and the peritoneal cavity) indicate that the mouse is not a suitable host for *F. gigantica*, in agreement with previous studies,[4] and that results obtained in mice should only be taken as suggestive.

Hamsters are, nevertheless, more appropriate hosts for *F. gigantica,*[4] and consequently, results obtained with this animal species offer more conclusive data. The number of hamsters infected by metacercariae kept in zeitoon-parvardeh was higher than that infected by metacercariae from delar. The percentage of worms recovered when using metacercariae from zeitoon-parvardeh was higher than that from delar, and the fluke number recovery when infecting with metacercariae kept in both foods was lower than that in water. All this suggests 1) a negative impact of the two foods on metacercarial infectivity and 2) a greater negative impact of delar than zeitoon-parvardeh.

When comparing results of microscopical analyses of metacercarial viability and results of experimental infection tests with mice and hamsters about metacercarial infectivity, there is an agreement in indicating a decrease of both viability and infectivity with time. Although it is already known that metacercarial age decisively influences both aspects in this way,[7] the results here obtained show that the two traditional foods analyzed seem to accelerate the decreasing processes of viability and infectivity of metacercariae. Moreover, data obtained indicate that delar has a greater negative impact than zeitoon-parvardeh on both metacercarial viability and infectivity, most probably because of the high salt concentration of delar.

In spite of this, results clearly prove that most metacercariae keep their viability and infectivity during the first two weeks after food preparation in both traditional specialties. Moreover, about half of the metacercariae keep their viability and infectivity in delar (salt = 40%; pH = 5) until at least four weeks. Thus, the present study shows the possible way of human contamination following the consumption of these two

TABLE 4
Effect of different conditions on the viability and infectivity of metacercariae of some foodborne trematodes

Conditions	Metacercariae	Time	Effects	Reference
Temperature (°C)				
–20	*Fasciola hepatica*	12 h	Non-infective	6
–10	*Fasciola hepatica*	7–28 d	Viable	6
–10 (in water)	*Fasciola hepatica*	7–28 d	Destroyed	6
–5	*Fasciola hepatica*	28 d	Infective	6
–5	*Fasciola hepatica*	56 d	Non-infective	6
–5 (12 h) and +10 (12 h)	*Fasciola hepatica*	70 d	Infective	6
–2	*Fasciola hepatica*	92 d	Infective	6
+10	*Fasciola hepatica*	130 d	Infective	6
+25	*Fasciola hepatica*	36 d	Infective	6
+30	*Fasciola hepatica*	14 d	Infective	6
+35	*Fasciola hepatica*	14 d	Died	6
+4	*Fasciola hepatica*	365 d	Survived	7
+22–31 in water (frequently changed)	*Fasciola hepatica*	80 d	Alive	35
+32 in water (exposed to sunlight)	*Fasciola hepatica*	30 d	Alive	35
Air (on leaves exposed to sunlight)	*Fasciola hepatica*	2–3 h	Died	35
Room temperature (shade)	*Fasciola hepatica*	72 h	Died	35
Air (12 h per day to direct sunlight)	*Fasciola hepatica*	2 d	Survived	36
Room temperature (shade)	*Fasciola hepatica*	17 d	Survived	36
+25–32 (dried on grass)	*Fasciola hepatica*	10 d	Died	37
–2	*Fasciola gigantica*	14 d	10% alive	6
	Fasciola gigantica	30 d	All died (microscopically)	6
+10	*Fasciola gigantica*	14, 54, 114 d	96, 92, 86% alive, respectively	6
+25	*Fasciola gigantica*	14, 54, 114 d	65, 81, 42% alive, respectively	6
+30	*Fasciola gigantica*	14, 54, 114 d	72, 16, 10% alive, respectively	6
+35	*Fasciola gigantica*	14, 54, 114 d	68, 6, 8% alive, respectively	6
Room temperature (in water)	*Fasciola gigantica*	180 d	Infective	38
+22–24 (detached leaves, indoors)	*Fasciola gigantica*	20 d	Survived	39
Plants exposed to daily sunlight (9.00am–3.00pm)	*Fasciola gigantica*	42 d	Survived	39
Plants in shade	*Fasciola gigantica*	13 d	Survived	39
Plants submerged in running water	*Fasciola gigantica*	122 d	Survived	39
Detached leaves in a quart jar	*Fasciola gigantica*	63 d	Survived	39
Boiling water	*Fasciolopsis buski*	1–2 min	Killed	30
Desiccation at 27°C	*Fasciolopsis buski*	19 h	Killed	30
–192 (liquid nitrogen)	*Clonorchis sinensis*	30 s	All killed	40
–12 (refrigerator)	*Clonorchis sinensis*	10 h	84% killed	40
–20	*Clonorchis sinensis*	3–7 d	Viable and infective	25
–12	*Clonorchis sinensis*	10–18 d	Viable and infective	25
–10	*Clonorchis, Opisthorchis*	5 d	Non-infective	41
–28	*Opisthorchis felineus*	32 h	Non-infective	41
–35	*Opisthorchis felineus*	14 h	Non-infective	41
–40	*Opisthorchis felineus*	7 h	Non-infective	41
–20	Heterophyid	8 h	Degenerated	24
Room temperature	Heterophyid	3 h	All degenerated	24
Acids				
10 ml/L citric acid	*Fasciola gigantica*	5–10 min	97–100% detached	12
1.0% hydrochloric acid	*Fasciolopsis buski*	18 d	Killed	28
2% acetic acid	*Fasciolopsis buski*	9 d	Killed	28
3% acetic acid	*Fasciolopsis buski*	6 d	Killed	28
5% and 10% acetic acid	Heterophyid	3 h	Degenerated	24
10% acetic acid	*Haplorchis taichui*	3 h	100% inactive but not degenerated	23
5% acetic acid	*Haplorchis taichui*	3 h	70% active	23
Salt				
5%	*Fasciolopsis buski*	3 h	Killed	28
Fish-salt (10 g/3 g)	*Clonorchis sinensis*	5–7 d	Viable and infective	25
5% and 10%	Heterophyid	3 h	Degenerated	24
10%	*Haplorchis taichui*	3 h	65% active	23
13.6%	*Opisthorchis*	24 h	Non-infective	22
Other salts				
24 mg/L potassium permanganate ($KMno_4$)	*Fasciola gigantica*	5–10 min	97.2–99.6% detached and lethal	12
24 mg/L potassium permanganate ($KMno_4$)	*Fasciola gigantica*	10 min	100% detached and 96% died	13
1 mg/L sodium dichloroisocyanurate (NaDCC)	*Fasciola gigantica*	15 min	100% detached and died	13
Fermentation				
Pla-Som (salted semi-fermented fish)	*Haplorchis taichui*	1 d	9.9% alive	23
	Haplorchis taichui	2 d	100% degenerated	23
Fish salad				
Lab-Pla (raw fish in spicy salad)	*Haplorchis taichui*	3 h	All active	23
Yum (fish in different ingredients)	Heterophyid	3 h	All degenerated	24

TABLE 4
Continued

Conditions	Metacercariae	Time	Effects	Reference
Irradiation				
Direct solar radiation	*Fasciolopsis buski*	20–30 min	Killed	29
0.15 KGy	*Clonorchis sinensis*	—	Effective for control	42
0.10 KGy	*Paragonimus westermani*	—	Unable to grow into adult worms in cat	43
2.5 KGy	*Paragonimus westermani*	—	No worm recovered from mice	43
200 KGy	*Metagonimus yokogawai*	—	Control of infectivity and development of parasites in rats	44
0.10 KGy	*Opisthorchis viverrini*	—	Non-infective	45
Other methods				
Running water	*Fasciola gigantica*	5–10 min	22.7–31% detached	12
120 ml/L commercial vinegar	*Fasciola gigantica*	5–10 min	100% detached	12
12 ml/L liquid soap	*Fasciola gigantica*	5–10 min	97–100% detached	12
Soybean sauce	*Fasciolopsis buski*	30 min	Killed	28
10% can sugar	*Fasciolopsis buski*	3 d	Killed	28

traditional food specialties of Gilan province, when prepared with raw vegetables presenting attached metacercariae. Results obtained indicate that the traditional methods of preparing these two local foods with fresh wild-grown plants offers little protection against contamination. The extended tradition of eating these foods in the Gilan Province, including a population of 2,000,000 people, may be a major source for human contamination with liver flukes.[1,9]

In Iran, human infection with fasciolids is mainly reported in this littoral region of the Caspian Sea, despite fascioliasis being prevalent in livestock throughout the country.[31,32] Only sporadic cases have been reported from other areas of the country.[33,34] Some of these sporadic cases may be linked to travelers who visit Gilan and sometimes take these foods as gifts.

Metacercariae treated with potassium permanganate. Even a low concentration of 24 mg/L of $KMnO_4$ was considered the most useful culinary tool to detach and kill metacercariae attached to various leaves and vegetables used for salads, in a short period of 5–10 minutes, without softening the leaves or changing their color, according to results obtained relatively recently.[12,13]

Unfortunately, results obtained in this study prove that potassium permanganate is not effective in killing metacercariae, even when applying doses much higher than those initially recommended (Table 3). Thus, potassium permanganate may be considered as a tool useful only for detaching metacercariae, similar to commercial vinegar (at a concentration of 120 mL/L), citric acid (10 mL/L), or liquid soap (12 mL/L). A 97–100% efficacy to detach metacercariae had been recognized for all these products.[12] The detaching capacity of potassium permanganate, by dissolving the external metacercarial attaching cement, was also observed in this study. Consequently, if it is used for such a purpose, careful, subsequent washing of leaves and vegetables with water is needed, as in the case of the above-mentioned products.

All evidence suggests that trematode metacercariae encysting in the outer environment, as those of fasciolids, are more resistant than those encysting in intermediate hosts (Table 4). In the case of *Fasciola* species, studies on the potential effectivity of different products and methods to kill metacercariae attached to leaves and vegetables are needed. Although appropriate studies should still be performed, metacercariae of *Fasciola* species may perhaps be more resistant than metacercariae of *F. buski*.[28–30]

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REFERENCES

1. World Health Organization, 1995. Control of foodborne trematode infections. *World Health Organ Tech Rep Ser 849:* 1–157.
2. Orlandi PA, Chu DMT, Bier JW, Jackson GJ, 2002. Parasites and the food supply. *Food Technol 56:* 72–81.

3. Mas-Coma S, Bargues MD, Valero MA, 2005. Fascioliasis and other plantborne trematode zoonoses. *Int J Parasitol 35:* 1255–1278.
4. Mas-Coma S, Bargues MD, 1997. Human liver flukes: A review. *Res Rev Parasitol 57:* 145–218.
5. Mas-Coma S, 2004a. Human fascioliasis. Cotruvo JA, Dufour A, Rees G, Bartram J, Carr R, Cliver DO, Craun GF, Fayer R, Gannon VPJ, eds. *World Health Organization (WHO), Waterborne Zoonoses: Identification, Causes and Control.* London, UK: IWA Publishing, 305–322.
6. Boray JC, Enigk K, 1964. Laboratory studies on the survival and infectivity of *Fasciola hepatica* and *F. gigantica* metacercariae. *Z Tropenmed Parasitol 15:* 324–331.
7. Valero MA, Mas-Coma S, 2000. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitol (Praha) 47:* 17–22.
8. Valero MA, Darce NA, Panova M, Mas-Coma S, 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Vet Parasitol 102:* 85–100.
9. Massoud J, 1990. Fascioliasis outbreak of man and drug test (Triclabendazol) in Caspian littoral, northern part of Iran, 1989. *Bull Soc Fr Parasitol 8:* 438.
10. Mas-Coma S, 2004b. Human fascioliasis: Epidemiological patterns in human endemic areas of South America, Africa and Asia. *Southeast Asian J Trop Med Public Health 35:* 1–11.
11. Mas-Coma S, 2005. Epidemiology of fascioliasis in human endemic areas. *J Helminthol 79:* 207–216.
12. El Sayed MH, Allam AF, Osman MM, 1997. Prevention of human fascioliasis: A study on the role of acids detergents and potassium permanganate in clearing salads from metacercariae. *J Egypt Soc Parasitol 27:* 163–169.
13. El-Zawawy LA, Ali SM, Allam SR, 2003. The effect of potassium permanganate and sodium dichloroisocyanurate on metacercariae of *Fasciola gigantica. J Egypt Soc Parasitol 33:* 305–314.
14. Mas-Coma S, Bargues MD, Valero MA, Fuentes MV, 2003. Adaptation capacities of *Fasciola hepatica* and their relationships with human fascioliasis: From below sea level up to the very high altitude. Combes C, Jourdane J, eds. *Taxonomy, Ecology and Evolution of Metazoan Parasites.* Volume II. Perpignan, France: Presses Universitaires de Perpignan, 81–123.
15. Moghaddam AS, Massoud JF, Mahmoodi M, Khoubbane M, Artigas P, Periago MV, Fuentes MV, Bargues MD, Mas-Coma S, 2004. Distributional outline of lymnaeid snails (Gastropoda) in the fascioliasis endemic area of Mazandaran, Iran. *Acta Parasitol 49:* 145–152.
16. Ashrafi K, Massoud J, Holakouei K, Mahmoodi M, Joafshani MA, Valero MA, Fuentes MV, Khoubbane M, Artigas P, Bargues MD, Mas-Coma S, 2004. Evidence suggesting that *Fasciola gigantica* might be the most prevalent causal agent of fascioliasis in northern Iran. *Iranian J Publ Health 33:* 31–37.
17. Boray JC, 1969. Experimental fascioliasis in Australia. *Adv Parasitol 8:* 95–210.
18. Esteban JG, Gonzalez C, Curtale F, Muñoz-Antoli C, Valero MA, Bargues MD, El Sayed M, El Wakeel A, Abdel-Wahab Y, Montresor A, Engels D, Savioli L, Mas-Coma S, 2003. Hyperendemic fascioliasis associated with schistosomiasis in villages of the Nile Delta, Egypt. *Am J Trop Med Hyg 69:* 429–437.
19. Dixon KE, 1966. The physiology of excystment of the metacercaria of *Fasciola hepatica* L. *Parasitology 56:* 431–456.
20. Tielens AGM, Van Der Meer P, Van Der Bergh SG, 1981. *Fasciola hepatica*: simple, large-scale, "in vitro" excystment of metacercariae and subsequent isolation of juvenile liver flukes. *Exp Parasitol 51:* 8–12.
21. Dixon KE, 1964. Excystment of metacercariae of *Fasciola hepatica in vitro. Nature 202:* 1240–1241.
22. Kruatrachue M, Chitramvong YP, Upatham ES, Vichasri S, Viyanant V, 1982. Effects of physico-chemical factors on the infection of hamsters by metacercariae of *Opisthorchis viverini. Southeast Asian J Trop Med Public Health 13:* 614–617.
23. Sukontason K, Methanitikorn R, Sukontason K, Piangjai S, Choochote W, 1998. Viability of metacercariae in northern Thai traditional foods. *Southeast Asian J Trop Med Public Health 29:* 714–716.
24. Wiwanitkit V, Nithiuthai S, Suwansaksri J, Chongboonprasert C, Tangwattakanont K, 2001. Survival of heterophyd metacercariae in uncooked Thai fish dishes. *Ann Trop Med Parasitol 95:* 725–727.
25. Fan PC, 1998. Viability of metacercariae of *Clonorchis* in frozen or salted freshwater fish. *Int J Parasitol 28:* 603–605.
26. Mas-Coma S, Angles R, Strauss W, Esteban JG, Oviedo JA, Buchon P, 1995. Human fasciolasis in Bolivia: A general analysis and a critical review of existing data. *Res Rev Parasitol 55:* 73–93.
27. Motawea SM, El Gilany A, Massoud A, Rizk H, El Shazly AM, Gaballah M, 2001. An epidemiological study of fascioliasis in a rural area in Dakahlia Governorate. *J Environ Sci (China) 21:* 31–62.
28. Komiya Y, 1964. Fasciolopsis buski. Morishita K, Komiya Y, Matsubayashi H, eds. *Progress of Medical Parasitology in Japan*. Tokyo: Meguro Parasitological Museum, 277–285.
29. Rim HJ, 1982. Fasciolopsiasis. Hillyer GV, Hopla CE eds. *Handbook Series in Zoonoses. Section C. Parasitic Zoonoses*. Volume III. Boca Raton, FL: CRC Press, 89–97.
30. Weng YL, Zhuang ZL, Jiang HP, Lin GR, Lin JJ, 1989. Studies on ecology of *Fasciolopsis buski* and control strategy of fasciolopsiasis. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 7:* 108–111.
31. Sahba GH, Arfaa F, Farahmandian I, Jalali H, 1972. Animal fascioliasis in Khuzestan, southwestern Iran. *J Parasitol 58:* 712–716.
32. Eslami A, Farsad-Hamdi S, 1992. Helminth parasites of wild boar, *Sus scrofa*, in Iran. *J Wildl Dis 28:* 316–318.
33. Mohsenin H, Ebrahimian MA, 1969. Human fascioliasis in Iran: Report of a case with *Fasciola hepatica* in biliary duct. *Bull Soc Pathol Exot 62:* 36.
34. Khorsandi HO, 1977. Obstructive jaundice due to *Fasciola hepatica*. Report of two cases. *Bull Soc Pathol Exot 70:* 626–628.
35. Shirai M, 1927. The biological observations on the cysts of *Fasciola hepatica* and the route of migration of young worms in the final host. *Sci Rep Gov Inst Infect Dis Tokyo 6:* 511–523.
36. Ross IC, Mckay AC, 1929. The bionomics of *Fasciola hepatica* in New South Wales and of the intermediate host *Limnaea brazieri* (Smith). *Bull Council Sci Indust Res Austral 43:* 1–62.
37. Ono Y, Isoda M, Matsumura S, 1954. I. Preventive study of *Fasciola hepatica* infection. II. Effects on metacercariae of various environmental conditions and drugs. *J Japa Vet Med Assoc 7:* 153–155.
38. Kimura S, Shimizu A, 1979. Viability of *Fasciola gigantica* metacercariae. *Nippon Juigaku Zasshi 40:* 357–359.
39. Alicata JE, 1938. Observations on the life history of *Fasciola gigantica*, the common liver fluke of cattle in Hawaii, and the intermediate host, *Fossaria ollula. Hawaii Agric Exp Station Bull 80:* 1–22.
40. Song SB, 1987. Larvicidal action of liquid nitrogen against metacercariae of *Clonorchis sinensis. Kisaengckunghak Chapchi 25:* 123–128.
41. WHO, 1979. Parasitic zoonoses. Report of a WHO Expert Committee with participation of FAO. *World Health Organ Tech Rep Ser 637:* 1–157.
42. Duan YF, Song CC, Shou GC, Zhu H, Shi JF, Fu JK, Zhang QY, 1993. Effect of gamma-irradiation on infectivity of *Clonorchis sinensis* metacercariae. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 11:* 45–49.
43. Song CC, Duan YF, Shou GC, Zhu H, 1992. Effect of cobalt-60 irradiation on the infectivity of *Paragonimus westermani* metacercariae. *J Parasitol 78:* 869–871.
44. Chai JY, Kim SJ, Kook J, Lee SH, 1995. Effects of gamma-irradiation on the survival and development of *Metagonimus yokogawai* metacercariae in rats. *Korean J Parasitol 33:* 297–303.
45. Sornmani S, Impand P, Bunditsing C, 1993. Irradiation of fish to control the infectivity of the liver fluke *Opisthorchis viverini*. Proceedings of the Final Research Co-ordination Meeting, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Mexico D.F., Mexico, June 24–28, 1991.