# Deliverable D-JRP22-WP3.6

## Workpackage WP3-T6

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**Contributing partners: COMSATS, ISS, NVI, UZ, RIVM, PIWET, UM, UN, IZS, UH, FFA, URCA, FLI, UT, BIOR, SSI, INSA.**

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# CONTAMINATION OF VEGETABLES FOR HUMAN CONSUMPTION BY EM/EG (MEME WP3-T6)

## INDEX

# CONTAMINATION OF VEGETABLES FOR HUMAN CONSUMPTION BY EM/EG

## 1. Introduction

### 1.1. Epidemiological context

Human infection by Em/Eg is caused by oral ingestion of microscopic parasite eggs. While the exact route of infection of individual human cases is generally unknown, the foodborne transmission is considered to be one the main route of infection as well as contact with dogs (Casulli & Tamarozi 2021). As eggs are inactivated by heat (i.e. cooking) the consumption of raw vegetables contaminated with viable eggs could lead to human infection, but today very scanty data are available on the degree of such contamination. Kitchen gardens was identified in France as hotspot for red fox and cat defecation resulting to higher exposure to Em eggs (Bastien et al., 2018).
The use of a robust and reliable method, coupling concentration of eggs and molecular biology for species identification recently published (Guggisberg et al., 2020) was validated for the detection of Em/Eg and other Taenidae eggs in lettuces and some others green leafy vegetables. A large multicentre study was organized in order to collect lettuces samples from different epidemiological situations in endemic areas for Em and/or Eg. The data obtained of the contamination of lettuces will contribute to a better understanding of human infections.

### 1.2. References


Bastien M, Vaniscotte A, Combes B, Umhang G, Germain E, Gouley V, Pierlet A, Quintaine T, Forin-Wiart MA, Villena I, Aubert D, Boue F, Poulle ML. Folia Parasitol (Praha). 2018. High density of fox and cat faeces in kitchen gardens and resulting rodent exposure to *Echinococcus multilocularis* and *Toxoplasma gondii*. doi: 10.14411/fp.2018.002.

Casulli A, Tamarozzi F. PLoS Negl Trop Dis. 2021. Tracing the source of infection of cystic and alveolar echinococcosis, neglected parasitic infections with long latency: The shaky road of "evidence" gathering. doi: 10.1371/journal.pntd.0009009.

Guggisberg AR, Alvarez Rojas CA, Kronenberg PA, Miranda N, Deplazes P. Pathogens. 2020. A Sensitive, One-Way Sequential Sieving Method to Isolate Helminths' Eggs and Protozoal Oocysts from Lettuce for Genetic Identification. doi: 10.3390/pathogens9080624.

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Petersen HH, Al-Sabi MNS, Enemark HL, Kapel CMO, Jørgensen JA, Chriél M. Parasitol Res. 2018. *Echinococcus multilocularis* in Denmark 2012-2015: high local prevalence in red foxes. doi: 10.1007/s00436-018-5947-y.

Trachsel D, Deplazes P, Mathis A. Parasitology. 2007. Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. doi: 10.1017/S0031182007002235.


## 2. Material and methods

### 2.1. Collection of samples

In order to evaluate the method of concentration of eggs, lettuces were purchased in supermarkets assuming that no eggs were present. These lettuces were spiked with known number of Em eggs

obtained from experimental infection of foxes with an Em strain (see Meme deliverable JRP18-WP1-T3).

In the context of the multicentre study, the lettuces and to a lesser extent some others green leafy vegetables were collected by 15 laboratory partners from 12 different countries (Figure 1). The samples were collected during summer 2021 while a preliminary collect in France was realized in summer 2020. It was proposed to each partner to collect 50 to 100 lettuces of preference from private kitchen gardens (2 from each origin) and local markets and in supermarkets (4 four each origin) if necessary to complete the sampling. Pools were constituted during the sequential sieving by grouping samples by two only if they have the same origin.

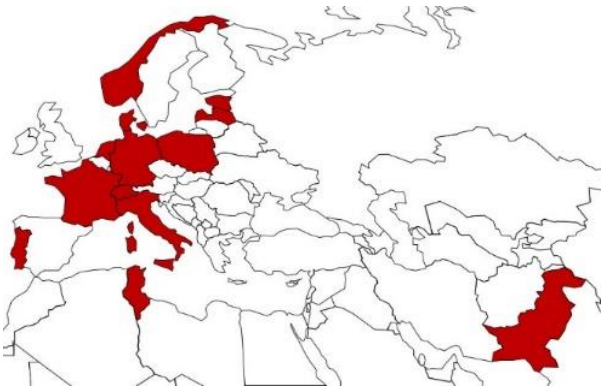

**Figure 1**: Geographical distribution of the 12 countries participating to the study of contamination of lettuces by Em/Eg and others Taenidae eggs.

## 2.2. Method of detection

The method to concentrate eggs was described by Guggisberg et al. (2020, Figure 2) consisting of a first washing step of 300g of lettuce leaves in plastic bag using Tween, followed by a sequential filtration using filter of different mesh size (105, 40 and 20µm). The last filter of 20µm is rinsed with Tween to collect the taeniid eggs and concentrate to obtain a pellet submitted to a tissue DNA extraction. Specific detection of Em and Eg sl was realized using dedicated real-time PCR from Knapp et al. (2016) and Maksimov et al. (2021), respectively. Detection of other *Taenidae* species was realized by a classical PCR (Trachsel et al., 2007) with identification of species after sequencing.

## 2.3. Validation of the method

As the method was not previously realised in the Anses laboratory, a validation was realized after it was transfer in this laboratory. First some preliminary tests with lettuces considered free of Em eggs and spiked with different quantities of Em eggs were processed and analysed by real-time PCR. After the ability of the technique was considered to be acquired, limit of detection was estimated by testing 24 lettuces for each quantity of eggs spiked.

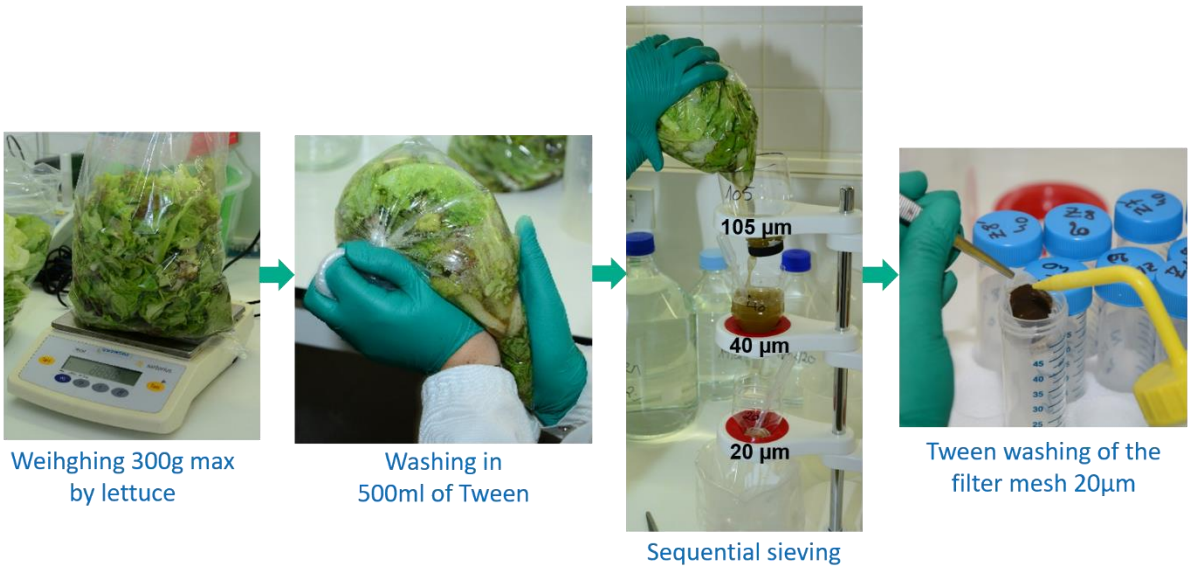


**Figure 2**: Visual description of the sequential sieving method to concentrate taeniid eggs from lettuce as realized at the Anses and previously published by Guggisberg et al., 2020.

### 2.4. Analyses of the multicenter samples

The transfer and validation of the method has revealed that the method needs some specific abilities and materials in order to maintain a relevant limit of detection. It was decided that the technique will be realised in only one laboratory (Anses) in order to assure homogenous assays and the correct limit of detection. As the lettuces samples need to be proceed in a short time after buying, the first washing step has to be realized in each participant's laboratory. So in addition to the SOP for the entire method, a dedicated SOP was provided concerning this first washing step. The pellets obtained after this washing step has to be grouped until the end of the collect and transferred to the Anses in order to realize the sequential sieving and molecular detection. For each lettuce sample, no leaves were excluded due to their appearance and no washing was realized before the one required for the protocol.
As most of the samples were analysed by pool of two samples from the same origin, it was assumed for positive pool that that only one of the two samples was considered as positive.

## 3. Results

### 3.1. Limit of detection of the method

Three batches of 24 lettuces each were spiked with 3, 2 or 1 Em eggs. The limit of detection (in 95% of the cases) was estimated to be at 3 eggs since the Em DNA was detected in 23 out of the 24 lettuces spiked with 3 eggs. Nevertheless, the detection of Em DNA was obtained in 75% (18/24) and 50% (12/24) for 2 and 1 eggs, respectively.

### 3.2. Detection from multicentre study samples

Among the 1,120 lettuces collected, 800 were originated from countries known to be endemic for Em (France, Switzerland, Denmark, Italy, Germany, Latvia and Pakistan). The Em DNA was detected in 7 cases from 4 countries corresponding to 1.1% (Table 1). In France, 2 cases were detected in the first collect in 2020 from 106 lettuces when 1 case was detected in 2021 from 122 lettuces. Additionally, one Em positive case was also detected in Germany from one chard sample.
Regarding Eg sl species, detection from lettuces was obtained in 23 cases corresponding to a global proportion of 2.1%. Eg ss was detected in the samples from the three areas of Italy (Roma, Napoli and Sardinia) corresponding to a global proportion of 3.5%. This species was also detected in high proportions in Pakistan (3%) and especially in Tunisia with detection of Eg ss DNA in 12% of the lettuces. If no case of *E. ortleppi* was observed, one case of *E. canadensis* was observed in Switzerland, Latvia and Pakistan. No case of Eg sl were obtained from vegetables others than lettuces.

Table 1: Detection of DNA from Em, Eg sl and other parasite species from lettuces and others green leafy vegetables from kitchen garden, local markets and supermarkets from different countries.

| | | France (Anses) | Portugal (Iniav) | Netherlands (RIVM) | Switzerland (UZH) | Denmark (ISS) | Germany | | Italy | | | Latvia (BIOR) | Poland (PIWET) | Norway (VETINST) | Pakistan (COMSTAT) | Tunisie (UM) | Total | Proportion detected: |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | (UH) | (FLI) | Roma (ISS) | Sardinia (IZS) | Napoli (UN) | | | | | | | |
| Lettuces | nb samples | 228 | 101 | 6 | 80 | 50 | 63 | 11 | 80 | 105 | 46 | 62 | 74 | 39 | 100 | 75 | 1120 | / |
| | nb of pools | 95 | 50 | 3 | 46 | 25 | 31 | 11 | 42 | 78 | 25 | 50 | 37 | 27 | 53 | 38 | 611 | / |
| | Em | **3** | 0 | 0 | **1** | **2** | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | **2** | 0 | **9** | **1%*** |
| | Eg sl | 0 | 0 | 0 | **1 *E. canadensis*** | 0 | 0 | 0 | **1 *Eg ss*** | **4 *Eg ss*** | **3 *Eg ss*** | **1 *E. canadensis*** | 0 | 0 | **3 *Eg ss* 1 *E. canadensis*** | **9 *Eg ss*** | **23** | **2,1%** |
| | other taenidae | 6 *Hydatigera sp.* | (1 *H. diminuta)* | 0 | 2 *Hydatigera sp.* | (1 *Dilepis undula)* | 1 *Hydatigera sp.* | 0 | 1 *Hydatigera sp.* | 3 *Hydatigera sp.*, 1 *T. multiceps* 1 *Taenia sp.* | 0 | 1 *Hydatigera sp.* (1 *Atriotaenia incisa)* | 0 | 1 *T. serialis* or *T. krabbei* | 5 *T. saginata,* 1 *Hydatigera sp.* 3 *T. hydatigena* | 3 *T. hydatigena* | 28 | 2,9% |
| Others vegetables | nb samples | / | / | / | 2 stem lettuces, 4 endive | / | / | 2 carrot leaves, 1 dandelion, 2 parsley, 6 chards | / | 1 chard | / | 1 coriander, 4 basil, 13 parsley, 2 celery leaves, 5 sorrel,1 kale, 10 spinach, 2 beet leaves | / | 11 chards | / | / | 67 | / |
| | Em | / | / | / | 0 | / | / | **1 (chard)** | / | 0 | / | 0 | / | 0 | / | / | **1** | / |
| | Eg sl | / | / | / | 0 | / | / | 0 | / | 0 | / | 0 | / | 0 | / | / | 0 | / |
| | other taenidae | / | / | / | 0 | / | / | 0 | / | 0 | / | 3 *Hydatigera sp.* (1 basil, 2 sorrel) | / | 1 *Hydatigera sp.* | / | / | 4 | / |

The proportion of others Taenidae species detected from lettuces was 2.9%. The species concerned was mainly *Hydatigera* sp. (15 cases), *T. hydatigena* (6) but also some cases of *T. multiceps*, *T. saginata* and *T. serialis* or *T. krabbei* since it was not possible to distinguish the two species with the sequence obtained. *Hydatigera* sp. was also detected from chard and in a pool of basil and sorel. Additionally, others parasite species were identified: *Hymenolepis diminuta*, *Dilepis undula* and *Atriotaenia incisa*.

## 4. Discussion

This multicentre study is the largest epidemiological study ever conducted on the contamination of vegetables by Em and Eg regarding the number of samples tested but also as the different origins mainly including European countries but also one from North Africa and another one from Asia. A high number of samples was required due to the low proportion of Em and Eg positive samples expected. The different origin was also relevant to cover different epidemiological situations.
The method was confirmed to be adapted to detection of taenid eggs in this epidemiological context with a relevant limit of detection of 3 eggs when one egg can be detected half of the time. The SOP provided to the participants for the washing step has also enabled to increase the number of participants while ensuring consistency and reliability of results.
Globally a high proportion of lettuces with taeniid eggs (5.4%) was detected. The dispersion of taeniid eggs from fecal sample to lettuces can be considered to be not a rare event. The contamination of lettuces by Em and Eg sl described in this study supports the importance of foodborne human infection.

Em was detected in high endemic areas in two consecutive collects in France (2020-2021) and in Switzerland (2019 from Guggisberg et al., 2020 and 2021 in this study) in the same proportions of around 1% allowing to establish that this proportion is a good estimation from high endemic areas of Western Europe. The absence of Em case from Poland and Germany can be explained more to be due to the sample size rather than to lower environmental contamination. The positive Em case of the chard identified in Germany reinforce this hypothesis. The absence of detection in Italy is not surprising as the areas concerned are not considered as endemic. The detection of two cases from lettuces in Denmark was not expected. If the lettuces were bought from supermarkets, one cannot discard the possibility than the lettuces were imported from an endemic countries. However, data about fox prevalence of Em in Denmark reported global very low prevalence but which can be reach higher level locally (Petersen et al., 2018). The detection in Pakistan is also interesting as few data are available concerning the presence of this species in the country where the role of dogs in direct or indirect (i.e. via vegetables) human infection may be more important as in other Asian countries than in Europe.
Regarding Eg sl species, all areas can be considered as endemic even if very low prevalence in definitive and intermediate hosts are reported from countries of Western Europe. The detection of Eg ss concerned countries know to be as high endemic areas as Italy, Tunisia and Pakistan. The high proportion of lettuces contaminated in this study confirm the foodborne route of infection for Eg ss when it is classically attributed to proximity with dogs. If the cases of *E. canadensis* from Pakistan and Latvia are in accordance with the reported presence of the species in these countries, it is not the case for Switzerland even if no recent data are available. The presence of this species correspond probably to a lifecycle between dogs and pigs.
The presence of *Hydatigera sp*. is associated with defecation of cats and was mainly observed in Europe. *T. hydatigena* was detected in high endemic areas for Eg ss which can be easily explain as it is associated to the same lifecycle between livestock and dogs. The case of *T. serialis* or *T. krabbei* from Norway can be explained by high proximity with wildlife as the intermediate hosts are lagomorphs and cervids, respectively. The definitive host of *T. saginata* is human, so the presence of these eggs on lettuces from Pakistan is indicative of poor sanitation. The presence of *T. multiceps* in Sardinia is well described and linked to high sheep breeding activity as for Eg ss.

Then presence of Em or Eg sl eggs on lettuces leaves represent a zoonotic threat. Nevertheless the data obtained in this study need to be interpreted with caution. The external leaves which are generally not consumed due to their bad appearance were not removed or they are the first to grow and with

greater proximity with the soil even if no data are available to confirm their higher risk of contamination. Secondly, the lettuces were not washed as they generally are before to be eaten but no information are available about efficiency of domestic washing to remove the taeniid eggs. Finally, if Em or Eg sl DNA was detected and can easily be assumed to come from eggs, there is no proof of infectivity of these eggs. In absence of molecular methods to estimate viability of the eggs, Guggisberg et al. (2020) has try for positive lettuces samples to inoculate half of the pellet obtained after sequential sieving to mice. No infection were obtained without constituting a proof of the absence of infectivity.

This large study about contamination of lettuces by Em and Eg sl eggs has confirm the potential foodborne source for both alveolar and cystic echinococcosis human cases. From these data, more are now needed to be obtained from others vegetables, from different type of berries as realised in an additional subtask of Meme (WP3T6ST2). This study also confirm the need to be able to evaluate infectivity of the eggs identify from food in order to go further in the risk of human infection evaluation.