Deliverable D-JRP22-WP3.6

Workpackage WP3-T6

Contamination of vegetables for human consumption by Em/Eg

Responsible Partner: Anses

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

GENERAL INFORMATION

European Joint Programme full title	**Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance and emerging microbiological hazards**
European Joint Programme acronym	**One Health EJP**
Funding	**This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.**
Grant Agreement	**Grant agreement n° 773830**
Start Date	**01/01/2018**
Duration	**60 Months**

DOCUMENT MANAGEMENT

Project deliverable	D-JRP22-WP3.6.1: Contamination of vegetables for human consumption by Em/Eg
Project Acronym	MEME
Author	Gérald UMHANG
Other contributors	
Due month of the report	M58
Actual submission month	M58
Type *R: Document, report* *DEC: Websites, patent filings, videos, etc.; OTHER*	R **Save date**: December 14, 2022
Dissemination level *PU: Public (default)* *CO: confidential, only for members of the consortium (including the Commission Services)*	PU
Dissemination *Author's suggestion to inform the following possible interested parties.*	OHEJP WP 1 ☐ OHEJP WP 2 ☐ OHEJP WP 3 ☒ OHEJP WP 4 ☐ OHEJP WP 5 ☐ OHEJP WP 6 ☐ OHEJP WP 7 ☐ Project Management Team ☐ Communication Team ☐ Scientific Steering Board ☐ National Stakeholders/Program Owners Committee ☐ EFSA ☐ ECDC ☐ EEA ☐ EMA ☐ FAO ☐ WHO ☐ OIE ☐ Other international stakeholder(s): …… Social Media: ..... **Other recipient(s):** .....

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

SUBTASK1: CONTAMINATION OF LETTUCES

SUBTASK2: CONTAMINATION OF BERRIES

SUBTASK3: DISPERSION OF EM/EG EGGS IN THE SOIL

INDEX

CONTAMINATION OF VEGETABLES FOR HUMAN CONSUMPTION BY EM/EG

1. Introduction

1.1. Epidemiological context

Human infection by *E. multilocularis* (Em) and *E. granulosus* (Eg) *sensu lato* 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 microscopic eggs are released in the environment by the carnivores definitive hosts mainly dogs and foxes via their feces. The presence of the eggs on the soil can result to contamination of food and water. Nevertheless, scarce data are available regarding the dispersion of the *Echinococcus* eggs from the feces to vegetables or water. Concerning Em, the environmental contamination by eggs is essentially due to red foxes in Western Europe (83 to 96%) with biotic potential resulting to the presence of 400,000 to 6.6 million eggs/km² depending of the density of foxes in rural or urban areas and their low or high prevalence level (Hegglin & Deplazes, 2013). This massive and heterogeneous environmental contamination of eggs, able to survive up to 240 days in autumn/winter period (Veit et al. 1995), is dispersed on the soil due to biotic (insects) and abiotic (wind, rain) factors.
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 (subtask 1) but also extended to berries (subtask 2). A large multicentre study was organized in order to collect lettuces samples and a second one for berries from different epidemiological situations in endemic areas for Em and/or Eg. The data obtained on lettuces and berries contamination of will contribute to a better understanding of human infections. The evaluation of the potential dispersion distance and main direction of Em eggs in an experimental context of a kitchen garden was realized in context of subtask 3.

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2. Material and methods

2.1. Collection of lettuces and berries samples

In order to firstly evaluate the method of concentration of the 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 maintain in mice in the Anses animal facility (see Meme deliverable JRP18-WP1-T3).

In the context of the first 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) only 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.

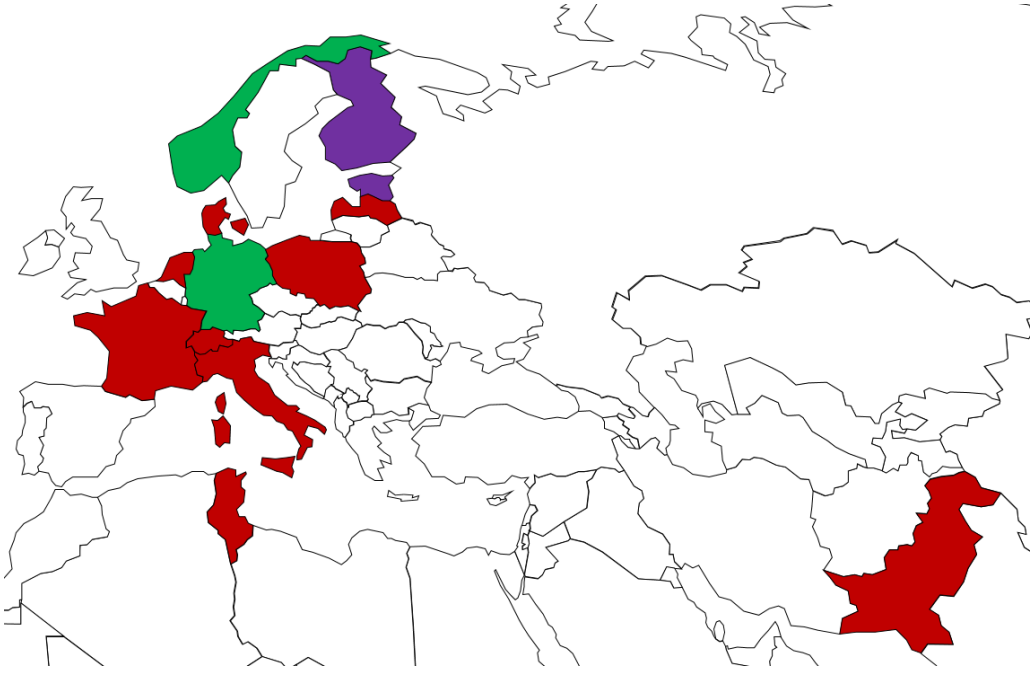

Figure 1: Geographical distribution of the 14 countries participating to the study of contamination of lettuces and berries by Em/Eg and others Taenidae eggs. Participants to both lettuces and berries studies are in red, only in lettuces in green and only in berries in purple.

The second multicentre study concerned mainly strawberries and blueberries, but others type of berries were also accepted. Twelve countries corresponding to 12 laboratories were concerned including two external partners to Meme which were the Finnish Food Authority from Finland and the University of Monastir from Tunisia. The collect was realized in 2022 targeting 20-30 berries samples (100-200g) country trying to prioritize local markets or directly from the wild and completed by supermarkets if needed. Unfortunately due to the severe flooding this summer, the collection of berries from Pakistan had to be postponed for several months and could not be received in time but the data will be added to the others when available.

2.2. Collection of soil samples after dispersion

A 2x2m naive soil plot was prepared outside (Figure 2). Feces from an experimentally infected foxes with an Em strain (see Meme deliverable JRP18-WP1-T3) were collected and submitted to flotation assay in order to evaluate the number of eggs per gram. A fecal sample containing 10,000 eggs was prepared and put on the centre of the soil plot and leaved during four months (July to October). At the end of the period, the soil plot was divided in 400 squares of 10x10cm and a soil sample (from the first 0-1 cm of soil) was collected from each. In the centre of the plot (12 squares of 10x10 cm), the soil was collected at two additional depth levels till 2-3 cm.

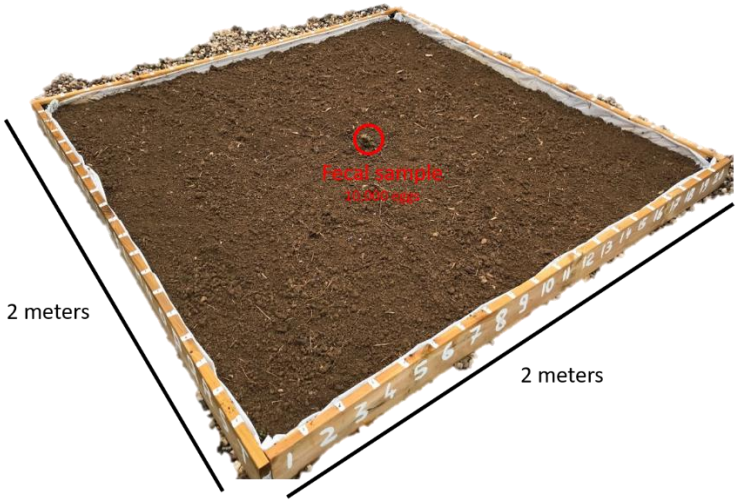

Figure 2: The native soil plot of 2x2m with the fecal sample containing 10,000 eggs in the centre.

2.3. Methods of detection

2.3.1. Detection of Em/Eg and other Taenidae species from lettuces and berries

The method to concentrate eggs from lettuces was described by Guggisberg et al. (2020, Figure 3). It consist to 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). For berries, the washing step was realized by placing the bag on an orbital shaker for 2x15 minutes at a rate that removes as much residue as possible from the berries by stirring the bags manually to mix the berries between the two sessions.

After the final filtration, the filter of 20µm is rinsed with Tween to collect the taeniid eggs and concentrated by centrifugation 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.

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.

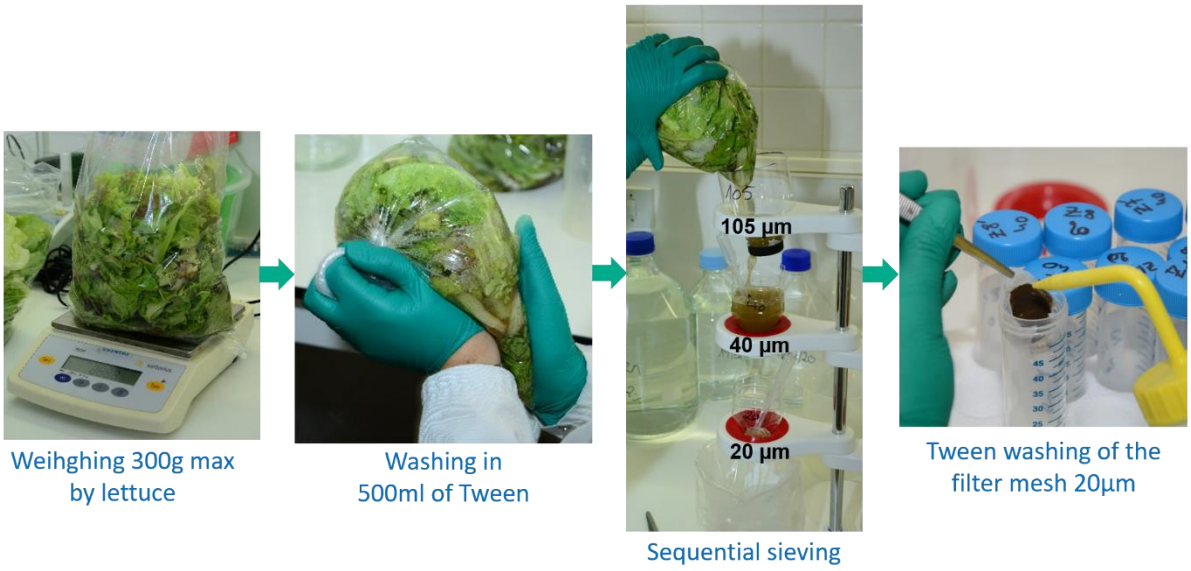

Figure 3: Visual description of the sequential sieving method to concentrate taeniid eggs from lettuce as realized at the Anses and previously published by Guggisberg et al. (2020).

2.3.2. Detection of Em from the fecal and soil samples

The fecal samples (5g) were submitted to a flotation technique based on sequential sieving initially published by Mathis et al. (1996) and slightly modified by Umhang et al. (2012). The numbers of eggs was evaluated by observation under microscope of the pellets obtained.
The soil samples were analysed using a flotation method previously described (Umhang et al. 2017) using 10g for each sample. The pellets obtained after flotation was submitted to an automatic DNA extraction using magnetic beads (Maxwell48). The detection of *E. multilocularis* DNA was realized by a real-time PCR targeting a short fragment of 12S mitochondrial gene (Knapp et al. 2016). An internal control is integrated to the qPCR in order to detect inhibition. A special qPCR mix (Maxwell GoTaqEnviro) was also used in order to be less sensitive to these potential inhibitors which are often present in soil samples.

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 in lettuces and another one for berries. 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 (maximum of 300g), no leaves were excluded due to their appearance and no washing was realized before the one required for the protocol. As most of the lettuces 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.
Concerning berries, one sample of 100 to 250g has to be obtain for each localization and has to be used without any other process. In some cases, two berries samples from the same type and localizations has been pooled during filtration and 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 for lettuces

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. Limit of detection of the method for strawberries

Three batches of 24 strawberries samples (200g 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 88% (21/24) for 2 and 1 eggs.

Table 1: Detection of DNA from Em, Eg sl and other cestode species in lettuces from kitchen gardens, local markets and supermarkets from different countries. *: the Em proportion was calculated including samples from known endemic countries. Cestode species other than Taenidae were indicated between brackets.

		nb of samples	Em	Eg sl	Other taenidae species
France (Anses)		228	**3**	0	6 *Hydatigera sp.*
Portugal (Insa)		101	0	0	(1 *H. diminuta)*
Netherlands (RIVM)		6	0	0	0
Switzerland (UZH)		80	**1**	**1 *E. canadensis***	2 *Hydatigera sp.*
Denmark (ISS)		50	**2**	0	(1 *Dilepis undula)*
Germany	**(UH)**	63	0	0	1 *Hydatigera sp.*
	(FLI)	11	0	0	0
Italy	**Roma (ISS)**	80	0	**1 *Eg ss***	1 *Hydatigera sp.*
	Sardinia (IZS)	105	0	**4 *Eg ss***	3 *Hydatigera sp.*, 1 *T. multiceps* 1 *Taenia sp.*
	Napoli (UN)	46	0	**3 *Eg ss***	0
Latvia (BIOR)		62	**1**	**1 *E. canadensis***	1 *Hydatigera sp.* (1 *Atriotaenia incisa)*
Poland (PIWET)		74	0	0	0
Norway (VETINST)		39	0	0	1 *T. serialis* or *T. krabbei*
Pakistan (COMSTAT)		100	**2**	**3 *Eg ss*** **1 *E. canadensis***	5 *T. saginata*, 1 *Hydatigera sp.* 3 *T. hydatigena*
Tunisie (UM)		75	0	**9 *Eg ss***	3 *T. hydatigena*
Total		1120	**9**	**23**	28
Proportion detected		/	**1%***	**2,1%**	2,9%

3.3. Detection from the lettuce multicentre study samples

Among the 1,120 lettuces collected, 674 were originated from countries known to be endemic for Em (France, Switzerland, Denmark, Germany, Latvia, Poland, Netherlands and Pakistan). The Em DNA was detected in 7 cases from 4 countries corresponding to 1.3% (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 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.
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*.

Table 2: Detection of DNA from Em, Eg sl and other cestode species from berries obtained from the wild, kitchen gardens, local markets and supermarkets from different countries. *: the Em proportion was calculated including samples from known endemic countries.

	strawberries	blueberries	raspberry	blackberries	others berries		Total nb of samples
France (Anses)	0/54	**1 Em**/31					85
Netherlands (RIVM)	**1 Em**/8	**1 Em**/6	0/3	0/3	0/4 redcurrant	0/1 white currant	25
Switzerland (UZH)	**1 Em**/10	0/7	0/6	0/6	0/1 redcurrant		30
Denmark (SSI)	**1 Em**/30 +1 Hydatigera sp.						30
Latvia (BIOR)	**4 Em**/30 +1 *T. serialis*	**4 Em** + **1 Eg ss**/30 +1 *M. melesi*					60
Estonia (UT)	**5 Em**/30						30
Poland (PIWET)	**1 Eg ss**/13	0/3	0/5	0/4	0/4 blueberry bushes	0/1 Canadian saskatoon berry	30
Italy-Sardinia (IZS)	**1 Eg ss**/34 +2 *Hydatigera sp.*						34
Portugal (Iniav)	**1 Eg ss**/17	0/15	0/15				47
Finland (FFA)	0/10	0/8	0/2		0/2 black currant	0/3 red cranberry	25
Tunisie (UM)	**13 Eg ss**/16 +1 *T. hydatigena*						16
Total nb of samples	252	100	31	13	16		**412**
Em proportion*	**Em: 6.9%**	**Em: 7.8%**					
Eg sl proportion	**Eg ss: 6.3%**	**Eg ss: 1%**					

3.4. Detection from the berries multicentre study samples

A total of 381 berries samples were collected mainly constituted by 252 strawberries samples and 100 blueberries samples but also of 8 others species (Table 2). The known Em endemic countries (France, Switzerland, Denmark, Netherlands, Estonia, Latvia and Poland) concerned 175 strawberries samples and 77 strawberries samples. Detection of Em DNA was realized in 12 cases corresponding to 6.9% in strawberries and 6 cases corresponding to 7.8% in blueberries from all known endemic countries excepting Poland. The two Baltic countries were mainly concerned with 13.3% (4 cases) in both strawberries and blueberries from Latvia and 16.6% (5 cases) in strawberries from Estonia. No case of Em were obtained from the others types of berries.

The detection of Eg sl DNA concerned 6.3% of strawberries and 1% of blueberries with absence of detection in others type of berries. It concerned four European countries with one case of Eg ss (Portugal, Italy-Sardinia, Latvia, and Poland) but mainly Tunisia with 13 cases among the 16 strawberries samples analysed corresponding to a very high proportion of 81.3%. If we exclude Tunisia, the proportion of Eg ss detection in strawberries bought in European countries is similar as the one for blueberries with 1.3%.

Regarding others Cestode species detected from berries, six cases were detected only from strawberries and blueberries. The definitive hosts of these parasite species concerned the cat (*Hydatigera sp*.), canids (*T. serialis* and *T. hydatigena*) but also badger (*Mescosetoides melesi*).

3.5. Dispersion of Em eggs in the soil

The remaining fecal sample from the centre of the plot was collected in order to estimate the number of eggs still present. A total of 378 eggs was estimated to be still present in the fecal sample corresponding to only 13% of the eggs which have not dispersed. The DNA of the eggs was detected in 39.5% of the soil samples that were distributed in all directions (Figure 4). The dispersion of eggs have reached the maximum distance of 1 to 1.4 m in the corners. The dispersion seems to have occurred mainly in the direction of the prevailing wind. According to Ct values of real time PCR obtained from soil samples and from individual eggs, a Ct threshold of 38.6 was established. Ct values inferior to this value were considered to correspond to more than one egg detected by qPCR after flotation of the 10g of soil sample and Ct values >38.6 correspond to only one egg. In 92% of the positive samples of 10g of soil, it was considered that only one egg was detected after the flotation. Moreover, in the centre of the plot (12 squares of 10x10 cm), the DNA of eggs was detected from three and four soil samples from 1-2cm (level -1) and 2-3 cm (level -2) from the surface, respectively (Figure 3).

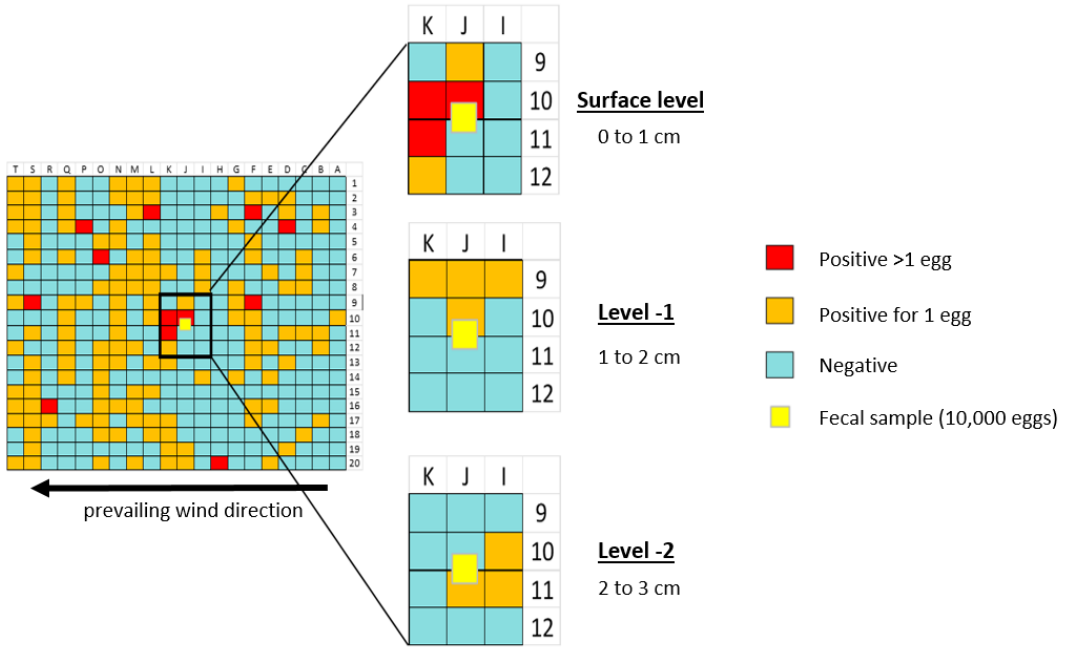

Figure 4: Spatial distribution after four months on the native soil plot of the soil samples with detection of Em DNA from one or more eggs from surface to 3 cm of depth.

4. Discussion

These two multicentre studies are the largest epidemiological study ever conducted on the food contamination 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. The different origins were also relevant to cover different epidemiological situations. A high number of samples was required due to the low proportion of Em and Eg positive samples expected. As berries can be more difficult to obtain the number of samples requested was lower.

The method was confirmed to be adapted to detection of taenid eggs in this specific epidemiological context with a relevant limit of detection of 3 eggs (in 95% of the cases) for both lettuces and strawberries with possibility to detect one egg in 50% and 88% of the cases, respectively. The choice to realize all the filtration and molecular analyses at the Anses by providing two dedicated SOP to the participants concerning sampling and the washing step for lettuces and berries has also enabled to increase the number of participants while ensuring consistency and reliability of results.

Globally a high proportion of food with taeniid eggs (5.4% in lettuces and 5.6% in berries) was detected. The dispersion of taeniid eggs from fecal sample to lettuces can be considered to be not a rare event. The results from subtask 3 highlights this potential of dispersion of taenid eggs especially as we should keep in mind that only 10g of soil was analysed for each sample which are constituted to around 100g each and that these estimation is also linked to the sensitivity of the methods especially flotation which can result to an underestimation of the number of positive squares and so of dispersion.
This can also explained why the detection in one square was not systematically realized at the three different levels of depth. The detection of eggs in depth is important as the eggs can be less exposed to desiccation and so have a better survival than those on the surface. Working the soil in a kitchen garden can then bring these eggs to the surface and contaminate vegetables. The subtask 3 task was successful to validate the experimental model to study the dispersion of Em eggs in the soil in the context of a kitchen garden. These first data have to be completed by additional ones and will be useful to understand dispersion of the eggs leading to contamination of vegetables.

Em was detected from lettuces 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 in lettuces from high endemic areas of Western Europe. The proportions of strawberries and blueberries with Em eggs are quite similar around 8% but higher than from lettuces from the same countries. Em contamination in Berries was detected in all the known endemic European countries sampled excepting Poland despite a lower number of samples reinforcing the hypothesis of a higher contamination rate than lettuces. Further data are need to clarify whether berries are more contaminated than lettuce because of their different appearance or growing locations. The absence of Em case from Poland (both lettuces and berries) and Germany (lettuces only) 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 but is in accordance with the case observed from strawberries. The lettuces were bought from supermarkets and strawberries from a local farm and all produced in the country. 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) and be source of lettuces contamination. A similar higher proportion of Em in berries from Latvia and Estonia were identified compared to others countries when prevalence in fox is reported to be similar or lower in these two Baltic countries. The same observation can be also realized from Netherlands. The detection in lettuces from Pakistan is also interesting as few data are available concerning the presence of this parasite species in the country where the role of dogs in direct or indirect (i.e. via vegetables) human infection may be more important than in Europe as in other Asian countries. Future data from berries will be interesting to observe if the proportion is also higher than from lettuces as observed 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 in lettuces concerned countries know to be as high endemic areas as Italy, Tunisia and Pakistan when it was also described in Latvia, Poland, and Portugal. The high proportion of lettuces and berries contaminated in this study confirm the foodborne route of infection for Eg ss when human infection may be classically attributed to proximity with dogs. If the lettuces 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. At the opposite in berries, detection of Eg ss cases in Poland and Latvia are surprising when E. canadensis is considered to be the most Eg sl important species as in eastern Europe.

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 and Latvia 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.

The contamination of lettuces and berries by Em and Eg sl eggs described in this study supports the importance of foodborne human infection due to dispersion of eggs from feces via the soil. Nevertheless the data obtained in this study need to be interpreted with caution. The external leaves of lettuces which are generally not consumed due to their bad appearance were not removed why 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. However, these considerations are not valid for berries since they are not washed and are eaten in its entirety. 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 available 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 especially due to the low sensitivity of the in vitro method.

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 including from others vegetables and type of berries. This study also confirm the need to be able to evaluate infectivity of the eggs identify from food in order to further assess the risk of human infection by Em and Eg sl.