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Food contamination by *Echinococcus* and other taeniid species: Typically low egg numbers, as estimated by digital PCR

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ABSTRACT

Echinococcus multilocularis and *Echinococcus granulosus* sensu lato are the causative agents of two major zoonotic diseases, alveolar echinococcosis and cystic echinococcosis, respectively, and are ranked among the four most important foodborne parasites worldwide. Although interest in food contamination by taeniid eggs is increasing, the estimation of the number of eggs present in food items is still lacking. Using *E. multilocularis* eggs isolated from fecal samples of experimentally infected red foxes, an average of 5261 (±1253) mitochondrial DNA copies per egg was estimated by digital PCR (dPCR). Based on this estimation, 47 taeniid DNA samples previously obtained

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from different food items and identified as positive for *E. multilocularis*, *E. granulosus* sensu stricto or *Taenia* species, were submitted to dPCR to estimate the number of eggs present. In 95.7% of the samples, the contamination was estimated to be caused by one to five eggs, with only one egg in 83.0% of the samples. As detection of such low-level contamination requires sensitive detection methods, a performance comparison between microscopy and molecular methods was conducted using 15 pellets obtained from lettuce wash residues spiked with 0 to 63 *E. multilocularis* eggs. Two operators performed blinded microscopy, followed by DNA extraction from the examined pellets for real-time PCR detection and then an estimation of the number of eggs by dPCR. A higher sensitivity was obtained with real-time PCR (88.5%) compared to microscopy (40%) in samples containing one to five eggs. The correlation between the number of eggs spiked into the samples and the estimated egg number was strong using dPCR ($r = 0.99$, $p < 0.01$), while only moderate with microscopy ($r = 0.67$, $p < 0.05$). Estimation of the number of taeniid eggs in food samples by dPCR provides the first quantitative basis for exposure assessment and is important for evaluating the risk of human foodborne infections, with future work needed to address egg viability.

1. Introduction

The Taeniidae family currently comprises four genera: *Taenia*, *Echinococcus*, *Versteria* and *Hydatigera* (Nakao et al., 2013). All the species have a dixenous lifecycle, with carnivores serving as definitive hosts. After the prepatent period, taeniid eggs are shed in large numbers via the feces into the environment. The egg production capacity of *Echinococcus* worms is considered lower than that of other taeniid species – a few hundred eggs compared with several tens of thousands, respectively (Alvarez Rojas et al., 2018). While only *Taenia solium* and *Taenia saginata* involve humans as definitive hosts, accidental ingestion of eggs from other taeniid species such as those of the *Echinococcus* genus can also lead to zoonotic diseases, as the larval stage may develop in humans. *Echinococcus multilocularis* and species of the *Echinococcus granulosus* sensu lato (*s.l.*) complex are the causative agents of alveolar echinococcosis (AE) and cystic echinococcosis (CE), respectively. Identifying the sources of human *Echinococcus* infections is challenging because of the long incubation period. The relative contribution of food-, water- and hand-to-mouth transmission remains unclear (Casulli and Tamarozzi, 2021; Tamarozzi et al., 2020; Torgerson et al., 2020). Nevertheless, both *E. multilocularis* and *E. granulosus* rank among the four most important foodborne parasites worldwide (FAO/WHO, 2014).

Over the last decade and on several continents, there has been an increase in the number of studies reporting the contamination level of food, such as fresh vegetables and berries, with eggs of *Echinococcus* spp. and other taeniid species (Barosi and Umhang, 2024; Saelens and Gabriël, 2020; Umhang et al., 2025). In terms of methodology, molecular tools targeting mitochondrial gene fragments (which have a higher copy number than the nuclear genome) have now largely replaced microscopic observation, which is considered fastidious, time-consuming, and less sensitive. The molecular approach is particularly relevant in the context of complex matrices characterized by low concentration of target DNA, such as feces and food samples.

The number of copies of mitochondrial genome DNA (mtDNA) in a single *Taenia hydatigena* egg has been estimated at around 7100 copies (±1900) using real-time PCR (Trachsel et al., 2007). Given their similar morphological characteristics, it was assumed that this number of mtDNA copies per egg could be extrapolated to other members of the Taeniidae family. Furthermore, it was hypothesized that fecal samples would contain parasitic DNA only from eggs. Based on these two assumptions, the value of 7100 mtDNA copies was used as a reference target for detection by copro-PCR and copro-real-time PCR assays in carnivore feces (Knapp et al., 2016; Maksimov et al., 2020; Trachsel et al., 2007).

However, accurate estimation of mtDNA copy number per egg is particularly important for zoonotic species such as *Echinococcus* spp., as it enables estimation of the number of eggs present in environmental and food samples. This estimation is especially important because quantitative data on the numbers of taeniid eggs in food samples, based on molecular methods, are currently unavailable. Low-sensitivity methods may overlook contamination with low egg numbers, so microscopy-based estimation is biased toward high egg counts, that are more readily observed, and thus may not reflect reality. In the absence of empirical data, it is currently considered that humans can become infected by ingestion of a single viable infectious egg, although exposure to larger numbers of eggs likely increases the probability of infection (Alvarez Rojas et al., 2018). Insight into the number of eggs present in food samples will therefore be useful for assessing the risk of human infections, as well as for guiding the selection of methods with an appropriate limit of detection in future studies.

Classically, the estimation of the mitochondrial or nuclear genome copy number has been based on real-time PCR, established by comparison of 10-fold dilutions of a standard DNA with a known number of target copies. The recently developed digital PCR (dPCR) method relies on end-point PCR in which the target DNA is partitioned into a very high number of individual amplification reactions (often >20,000). The total number of reactions and the number of positive reactions are then used to determine the absolute quantity of the target DNA, calculated according to Poisson's distribution (Vogelstein and Kinzler, 1999). Unlike real-time PCR, dPCR does not rely on a calibration curve. This characteristic enables dPCR to achieve a lower detection limit, greater precision, and increased robustness against potential inhibitors present in the reaction compared with real-time PCR (Huggett, 2020). The dPCR method has already been used in human and veterinary parasitology for protozoa (e.g., *Plasmodium falciparum* and *Toxoplasma gondii*), nematodes (mainly *Haemonchus* sp.) but also for cestodes (Baltrušis and Höglund, 2023; Pomari et al., 2019). Focusing on the Taeniidae family, dPCR has been used for the detection, but not the quantification, of DNA from eggs of *T. solium* in soil samples (Maganira et al., 2019),

as well as DNA from *E. multilocularis* or *E. granulosus* s.l. in human blood, rodent liver or dog fecal samples (Bagó et al., 2021; Baraquin et al., 2018; Jiang et al., 2025; Massolo et al., 2021; Selcuk et al., 2025).

In this study, dPCR was used to quantify the number of mtDNA copies per egg in the two major *Echinococcus* species (*E. multilocularis* and *E. granulosus* s.s.), as well as in *T. hydatigena*. The resulting reference values of mtDNA copies were then applied to estimate the number of eggs present in food samples contaminated with taeniid eggs identified in a previous study. Based on these data, a qualitative and quantitative performance comparison was conducted between microscopic and molecular (real-time PCR and dPCR) methods for the detection of *E. multilocularis* eggs spiked into food pellets. The main objective of this comparison was to identify the most reliable and sensitive approach for detecting and quantifying taeniid eggs in food.

2. Materials and methods

2.1. Origin of taeniid eggs used for mitogenome copy number estimation

Eggs of *E. multilocularis* were obtained from the feces of two red foxes (*Vulpes vulpes*) experimentally infected with a Swiss isolate maintained through several passages in mice (Fig. 1). In both foxes, the presence of *E. multilocularis* eggs was first confirmed using the Mini-FLOTAC technique (Cringoli et al., 2017; Nocerino et al., 2024a). From the first fox (referred to in the manuscript as the 'first infection'), two batches of eggs were prepared: (i) fresh feces stored at 4 °C without prior inactivation, with viability assessed by sodium hypochlorite treatment (Alvarez Rojas et al., 2018); and (ii) feces stored for one week at −80 °C for inactivation and subsequently at −20 °C. From the second fox ('second infection'), eggs were isolated from feces stored directly at −80 °C. Eggs of *E. granulosus* s.s. and *Taenia hydatigena* were obtained from two sheep-dog fecal samples collected in Greece within the Echino-Safe-Med project (Nocerino et al., 2024a; Nocerino et al., 2024b). These samples were stored at −80 °C for one week and underwent repeated thawing in the context of previous analyses (Maksimov et al., 2020; Nocerino et al., 2024a; Trachsel et al., 2007), before a final thawing for individual egg isolation in the present study.

2.2. Isolation and species confirmation of taeniid eggs in fecal samples

Between 200 mg and 1 g of each fecal sample was diluted in distilled water and filtered through a 105 µm mesh followed by several washing and centrifugation steps (Fig. 1). After removing the supernatant, the pellet was examined under a stereoscopic microscope (x50–100). Taeniid eggs were individually isolated using a home-made micropipette fashioned from a streamlined heparinized capillary tube, allowing the isolation of up to 10 eggs per fecal sample. The isolated eggs were individually submitted to DNA extraction (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany) after overnight chemical lysis with proteinase K under recommended conditions. Real-time PCR assays were performed to confirm the species of the eggs for *E. multilocularis* (Isaksson et al., 2014)

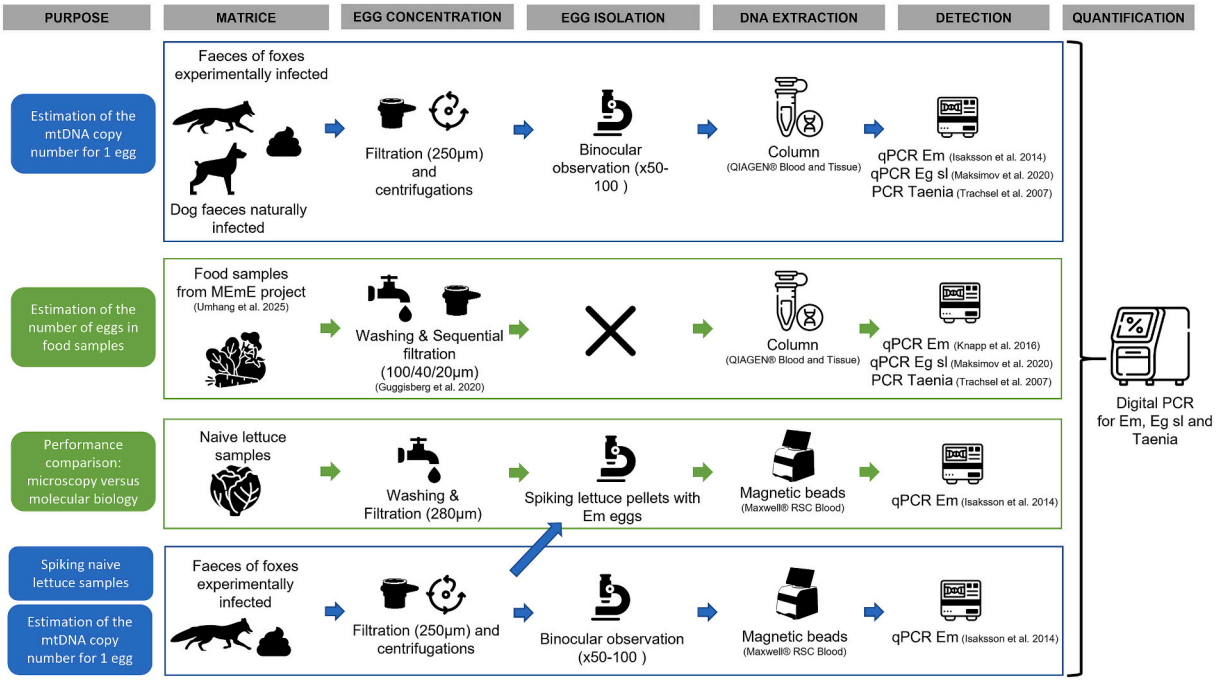

Fig. 1. Schematic representation of the workflow used for each type of matrix, based on the initial objective, from egg concentration to the estimation of mitochondrial DNA (mtDNA) copy number by dPCR.

and *E. granulosus* s.s. (Maksimov et al., 2020). For *T. hydatigena*, the species was confirmed by PCR and sequencing of a short *cox1* fragment (Bowles et al., 1992).

2.3. Digital PCR

2.3.1. Digital PCR conditions

The dPCR assays were performed on a QIAcuity One instrument (QIAGEN, Hilden, Germany), with 26,000-partition nanoplates (26 K, 24-well format), and the results were analyzed using the QIAcuity Suite software version 2.5.0.1. Three specific dPCR reactions were performed to detect and quantify *E. multilocularis*, *E. granulosus s.s.*, and *Taenia* spp. using the primers and probes described in Table 1. All dPCR assays were optimized to achieve the best differentiation between positive and negative scatter plots, in order to ensure reliable quantification. Each reaction mixture contained 10 µL of QIAcuity Probe PCR Master Mix 4× (QIAGEN, Hilden, Germany), primers at a final concentration of 2 µM, and a probe at a final concentration of 0.4 µM. To minimize steric hindrance during the amplification of large DNA fragments (>20 kb), the supplier recommends the addition of a restriction enzyme. Therefore, 1 µL of *Hin*fI (0.25 U/µL) was added to the reaction mixture for the *E. multilocularis* assay, 0.5 µL of the same enzyme for the *E. granulosus s.s.* assay, and 1 µL of AccII (0.25 U/µL) for the *Taenia* spp. dPCR assay. After adding the DNA obtained from the food sample to the reaction mixture, the nanoplates were incubated for 10 min at room temperature to allow enzymatic digestion. The final reaction volume was 40 µL per well, including 5 µL of DNA template. Negative and positive controls were systematically included for each series of analyses. The thermal cycling conditions were the same for the *E. multilocularis* and *E. granulosus s.s.* assays: an activation step at 95 °C for 2 min, followed by 45 cycles of 15 s at 95 °C and 30 s at 60 °C. For the *Taenia* spp. dPCR assay, the activation step at 95 °C for 2 min was followed by 50 cycles of 15 s at 95 °C and 60 s at 53 °C. Image acquisition on the QIAcuity One instrument was set to 300 ms exposure and a gain of 4 for both *E. multilocularis* and *E. granulosus s.s.* dPCR assays, and to 500 ms with a gain of 6 for the *Taenia* spp. dPCR assay. The results generated by QIAcuity are reported as the number of DNA copies per microliter of reaction. The total number of mtDNA copies present in a single egg was calculated from the dPCR results according to the following formula:

$$\text{nb mtDNA copies in on egg} = \frac{\text{nb copies per } \mu\text{L} \times \text{total reaction volume } (40\ \mu\text{L}) \times \text{Elution volume } (200\ \mu\text{L})}{\text{volume of DNA for dPCR reaction } (5\ \mu\text{L})}$$

The number of eggs present in food sample was then estimated by dividing the total number of mtDNA copies obtained by the average number of mtDNA copies estimated in one taeniid egg.

2.3.2. Limit of blank (LoB)

The positivity threshold for each dPCR assay was established based on the limit of blank (LoB) determined for each combination of the three dPCR assays and the two matrices analyzed in this study (lettuce and berries). For each matrix–assay combination, at least 30 reactions were performed by dPCR on negative samples according to previous real-time PCR results (Umhang et al., 2025). The maximum number of positive partitions observed in target-free samples was used to define the LoB. To ensure analytical rigor and limit the risk of false positives, the positivity threshold was defined as the maximum number of positive partitions observed in a negative sample plus one additional positive partition.

2.4. Estimation of the number of eggs in contaminated food samples

The estimation of egg number in food samples was performed using DNA collected and analyzed in two previous multicentre studies of the One Health European Joint Programme MEmE (Multi-centre study on *Echinococcus multilocularis* and *Echinococcus granulosus s.l.* in Europe: development and harmonisation of diagnostic methods in the food chain) project, which involved 12 European countries as well as Tunisia and Pakistan (Umhang et al., 2025). The food samples, including lettuce, strawberries and

Table 1
Nucleotide sequences of primers and probes used for the three dPCR assays.

Parasite species	Assay name (Gene Targeted)	Primer/ Probe	Oligonucleotide sequences (5′-3′)	Product size (bp)	Origiginal references of the real-time PCR assays
E. multilocularis	Em_MGB dPCR (*rrnS*)	EmMGB_F EmMGB_R EmMGB_P	GTGCTGCTYATAAGAGTTTTTG CTATTAAGTCCTAAACAATACCATA FAM-ACAACAATATTCCTATCAATGT-MGB	77	Isaksson et al. 2014
E. granulosus s.s.	Eg_ss dPCR (*cox1*)	Eg_G1-3_cox1_7122_F Eg_G1-3_cox1_7230_R Eg_G1-3_cox1_7149_P	AGGGGCTGGTGTTGGTTGGA TGAAACACCAGCCAAATGCAGAGA FAM -TCC GCC GTT GTC CTC GTC GT-BHQ1	109	Maksimov et al., 2020
Taenia spp.	*Taenia* spp. dPCR (*rrnS*)	Cest_F Cest_R Tspp_MGB_Probe	AGTCTATGTGCTGCTTAT CCTTGTTACGACTTACCT FAM-ATGCGTTACWTT-MGB	179	Oberli et al., 2023

blueberries, were previously analyzed using a sequential sieving method (Guggisberg et al., 2020) followed by DNA extraction with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Molecular detection was then performed using real-time PCR for *E. multilocularis* (Knapp et al., 2016) and for *E. granulosus* s.l. (Maksimov et al., 2020), and classical PCR followed by sequencing for other taeniid species (Trachsel et al., 2007) (Fig. 1). From the total DNA sample set, a selection of 47 taeniid positive DNA samples for

Table 2
Estimation of the number of mitochondrial genome copies for a single egg obtained by digital PCR after DNA extraction using the DNA Blood & Tissue kit (Qiagen), according to the taenidae species, host species, origin and batches. * Nb of mtDNA copies for 1 egg = Conc. [cp/µL] dPCR reaction X 40 µL (total dPCR reaction volume) / 5 µL (DNA input volume) X 200 µL (DNA elution volume).

Fecal samples ID	Egg ID	Country of origin	Host species	Parasite species	Conc. [cp/µL] dPCR reaction (undiluted sample)	Valid partitions	Positive partitions	Nb of mtDNA copies for 1 egg *	Average nb of mtDNA copies for 1 egg by batch (standard deviation)
EJPa (−80 °C)	1	France	captive red fox	*E. multilocularis*	2.15	25231	40	3435	5592 (±1382)
	2				4.18	25462	78	6685	
	3				3.66	25452	70	5854	
	4				2.05	25474	38	3277	
	5				4.41	25342	80	7056	
	6				3.66	25427	66	5862	
	7				4.49	25212	84	7178	
	8				2.90	25447	54	4645	
	9				3.72	25468	69	5958	
	10				3.74	25447	69	5979	
EJP17 (−80 °C)	1	France	captive red fox	*E. multilocularis*	3.51	25359	65	5608	5244 (±1438)
	2				4.02	25456	75	6435	
	3				3.71	25408	69	5.935	
	4				1.45	25457	27	2318	
	5				4.04	25453	73	6.463	
	6				2.92	25484	53	4.674	
	7				3.30	25462	59	5.275	
EJP17 (viable)	1	France	captive red fox	*E. multilocularis*	4.09	25468	76	6539	4940 (±1005)
	2				3.16	25462	59	5057	
	3				3.84	25413	72	6144	
	4				3.08	25465	59	4927	
	5				3.00	25389	57	4793	
	6				2.64	25452	49	4231	
	7				1.87	25489	34	2993	
	8				2.73	25470	49	4369	
	9				3.48	25471	63	5572	
	10				2.98	25426	54	4774	
D188	1	Greece	sheep dog	*E. granulosus s. s.*	2.93	25393	54	4694	3445 (±1179)
	2				2.73	25363	50	4362	
	3				1.92	25415	36	306	
	4				2.41	25433	45	3860	
	5				1.86	25473	35	2978	
	6				1.57	25460	30	2504	
	7				0.84	25451	16	1341	
	8				1.67	25464	31	2674	
	9				2.26	25449	41	3615	
	10				3.35	25450	60	5356	
D15	1	Greece	sheep dog	*T. hydatigena*	2.68	25449	49	4282	3017 (±1495)
	2				2.64	25362	50	4217	
	3				0.75	25422	14	1193	
	4				3.39	25415	63	5422	
	5				1.65	25458	31	2635	
	6				1.01	25463	19	1621	
	7				1.35	25411	25	2154	
	8				1.02	25443	19	1629	
	9				2.50	25457	47	4002	
D63	1	Greece	sheep dog	*T. hydatigena*	0.98	25444	18	1569	3321 (±1120)
	2				2.21	25462	40	3530	
	3				1.86	25461	34	2982	
	4				2.73	25446	51	4365	
	5				2.60	25430	49	4159	
D61	1	Greece	sheep dog	*T. hydatigena*	1.41	25456	27	2254	3418 (±2399)
	2				4.57	25443	87	7304	
	3				2.37	25472	44	3796	
	4				0.61	25463	11	969	
	5				1.73	25443	31	2766	

E. multilocularis, *E. granulosus* s.s. and *Taenia* spp. from different food items was made. These DNA samples were used in this study to estimate the number of eggs using the dPCR approach.

2.5. Qualitative and quantitative comparison of microscopy and molecular methods for detecting taeniid eggs in food samples

Batavia lettuces were purchased from supermarkets in France, to obtain negative food pellets (Fig. 1). For each lettuce, 300 g of leaves were put in a blender bag (Interscience) with a full-screen microperforated filter (porosity 280 µm). After addition of 500 mL of PBS-Tween solution and removal of the air from the inside, the stomacher bag was hermetically closed and shaken horizontally for 10 min (5 min each side) on an orbital shaker, followed by 1 min of manual shaking. The filtered washing solution was transferred to a tube for three successive centrifugation steps, with the supernatant removed each time and the tube refilled with water. The washing pellets obtained from each sample were pooled together and stored at 4 °C. As the average volume of a washing pellet obtained from 300 g of lettuce leaves is about 200 µL, 15 pellets of this volume were individually placed in Petri dishes spiked with 0 to 63 *E. multilocularis* eggs obtained from experimentally infected foxes, as described above. Each pellet was then examined under a stereoscopic microscope and the eggs counted by two blinded operators experienced in taeniid egg identification.

As the proportion of contaminated food samples is generally low, requiring the processing of a large number of samples, the time for microscopic examination was limited to approximately one hour per sample for each operator to replicate routine testing conditions. After microscopic examination, the content of the Petri dish was collected in a 50 mL tube for centrifugation. The pellets were subjected to DNA extraction using the Maxwell® RSC Blood DNA kit with the Maxwell48 RSC automatic extractor (Promega) following the manufacturer's recommendations, but with an overnight chemical lysis step. A real-time PCR for detection of *E. multilocularis*, followed by dPCR estimation of egg number, was performed as described above.

2.6. Mitochondrial DNA copy number per E. multilocularis egg extracted with magnetic beads

The DNA extraction method (i.e., magnetic beads versus a silica-membrane-based column) was expected to have an impact on the estimated copy number by dPCR due to differences in DNA yields between methods. As magnetic beads were used in the food sample experiments comparing microscopy and molecular methods, it was necessary to determine the number of mitochondrial copies detected per *E. multilocularis* egg using this extraction method (Fig. 1). In this context, 15 additional lettuce pellets obtained by the same washing and filtration procedure as the pellets used for microscopic observation, were spiked with 1, 2, 3, 5 or 10 *E. multilocularis* eggs from the same experimental infection and processed by automatic DNA extraction using magnetic beads, followed by dPCR. The number of mtDNA copies per egg was calculated from the dPCR concentration, taking into account a total dPCR reaction volume of 40 µL, an initial DNA volume of 5 µL and an elution volume of 100 µL. An average value for the number of mtDNA copies per *E. multilocularis* egg, obtained in this specific technical context, was used for the method comparison. Additionally, a comparison with results obtained using a silica column-based DNA extraction method was possible.

2.7. Statistical analyses

The correlation between the number of eggs spiked into the pellets and the number of eggs observed by microscopy or estimated by dPCR was calculated using the Pearson correlation coefficient. The correlation coefficient ranges from 0 to 1.0, where a correlation coefficient of zero denotes independence between the variables and a value of 1 is a perfect correlation. Correlation plots were also created to visualize the number of eggs spiked into the pellets versus the results obtained with microscopy or dPCR, including 95%

Table 3
Estimation of the number of mitochondrial genome copies for a single egg of *E. multilocularis* obtained by digital PCR from a washing pellet obtained after washing and filtration of 300 g of lettuce leaves followed by magnetic DNA extraction. * Nb of Total mtDNA copies = Conc. [cp/µL] dPCR reaction X 40 µL (total dPCR reaction volume) / 5 µL (DNA input volume) X 100 µL (DNA elution volume).

	digital PCR *E. multilocularis*					
Nb of egg spiked	Conc. [cp/µL] (dPCR reaction)	Valid partitions	Positive partitions	Nb of Total mtDNA copies *	Nb of mtDNA copies for one egg	
10	19.236	25451	347	15389	1.539	19.236
5	9.279	25452	166	7423	1.485	9.279
5	12.189	25375	219	9751	1950	12.189
3	6.630	25460	120	5304	1768	6.630
3	10.454	25465	191	8363	2788	10.454
3	8.774	25449	165	7019	2340	8.774
2	5.166	25385	98	4133	2067	5.166
2	1.972	25410	37	1578	789	1.972
2	6.294	25439	117	5035	2518	6.294
1	0.710	25392	13	568	568	0.710
1	3.356	25393	62	2685	2685	3.356
1	1.591	25437	29	1273	1273	1.591
1	2.410	25412	45	1928	1928	2.410
1	0.848	25433	16	678	678	0.848

confidence intervals.

The overall performance of microscopy and dPCR methods for estimating the number of *E. multilocularis* eggs in food samples was compared using the mean absolute error, calculated as the difference between the estimated and the spiked number of eggs. All statistical analyses were performed using R software (R. version 4.4.1).

3. Results

3.1. Mitogenome copy number per taeniid egg

In the first step, the number of mtDNA copies in a single taeniid egg was estimated following DNA extraction using the DNA Blood & Tissue kit (Qiagen, Hilden, Germany), a column-based method. For *E. multilocularis*, the estimated number of mtDNA copies ranged from 2318 to 7178 among the 27 eggs tested, with batch averages of 4940 (±1005) to 5592 (±1382) (Table 2). Values obtained for fresh viable eggs were similar to those of the other two batches. Considering the three batches, the average number of mtDNA copies per *E. multilocularis* egg was estimated at 5261 (±1253). Lower numbers of mtDNA copies, but similar within species, were obtained from the 10 eggs of *E. granulosus* s.s. which averaged 3445 (±1179) copies, and from three batches comprising 19 eggs of *T. hydatigena* which ranged from 3017 (±1495) to 3418 (±2399) copies, with an overall mean of 3203 (±1608) copies.

On the other hand, the average number of mtDNA copies per *E. multilocularis* egg obtained from a washing pellet of 300 g of lettuce leaves, using a magnetic bead-based DNA extraction protocol, was estimated at 1741 (±727) based on values obtained from one to 10 eggs (Table 3). This value is much lower than that obtained for *E. multilocularis* using the column-based protocol.

3.2. Determination of the limit of blank (LoB) in food samples

The LoB was determined for each combination of the three dPCR assays and the two food matrices (Table 4). The positivity thresholds ranged between one and four positive partitions per reaction, depending on the assay and matrix. These thresholds were applied to all subsequent dPCR measurements to distinguish true positive signals from background noise. Furthermore, all dPCR reactions showed more than 25000 valid partitions per well (out of 26000), confirming the technical validity of the measurements.

3.3. Estimation of the number of taeniid eggs in food samples

Regarding the results obtained for the number of mtDNA copies in one taeniid egg isolated from fecal samples, variations in sample storage conditions may have influenced DNA preservation and quantification. Additionally, as taeniid eggs are already infectious after being shed in the feces, the number of mtDNA copies is not expected to increase, and the highest values may be closest to the real number of mtDNA copies. Therefore, the number of eggs in food samples was systematically estimated using the value obtained for *E. multilocularis* from fresh material derived from experimental infections (i.e., 5261 copies), regardless of the Taeniidae species.

According to the dPCR assays, the number of eggs estimated to be present in the 47 DNA food samples previously extracted using the DNA Stool Kit (Qiagen, Hilden, Germany) that tested positive for *E. multilocularis*, *E. granulosus s.s.* or other taeniid species ranged from one to five in 95.7% of the samples (Table 5). In 83% of the positive food samples, contamination was attributed to a single egg. Only two samples contained more than five eggs, with estimates of eight and ten eggs, respectively.

3.4. Comparative performance of microscopy and molecular tests for detecting taeniid eggs in food samples

Each sample was microscopically examined by two operators for 50 to 90 min, with an average examination time of 68 min. The sample containing no eggs was correctly identified as negative by both operators (Table 6). Six samples containing between one and eight eggs were also scored negative by both operators, whereas two samples (containing five and 21 eggs) were positive for operator 1 but negative for operator 2. Eggs were observed by both operators in the remaining six samples. There was a moderate correlation between egg counts estimated by the two operators (Pearson's $r = 0.54$, $p < 0.05$). As the agreement was not perfect, the highest egg count recorded by either operator for each sample was retained for comparison with the molecular methods. With regard to dPCR, the estimated egg number generally corresponded well with the number of eggs spiked into the samples. For the sample containing no eggs, a single positive signal was observed; however, this signal was below the LoB and was therefore not considered positive.

The sensitivity of detection of taeniid eggs for the 14 spiked food pellet samples was estimated at 57% using microscopy (8/14

Table 4
Positivity thresholds depending the combination of between the digital PCR assays and the type of food matrix in order to estimate the limit of blanks.

Parasite species targeted	Digital PCR assay	Food matrix	Maximum positive partitions observed	Corresponding LOB (copies/µL)	Threshold for positivity
E. multilocularis	Em_MGB	Lettuces	1	0.427	≥2 positive partitions
		Berries	2	0.856	≥3 positive partitions
E. granulosus s.s.	Eg_ss	Lettuces	0	0	≥1 positive partition
		Berries	1	0.397	≥2 positive partitions
Taenia spp.	Taenia_spp	Lettuces	2	0.586	≥3 positive partitions
		Berries	3	0.237	≥4 positive partitions

Table 5
Estimation by digital PCR of the number of eggs contaminating the food samples, after column-based DNA extraction, previously identified from the two multicentre studies of the MEmE project. Number of eggs superior to one are in bold. * Nb of mtDNA copies = Conc. [cp/µL] dPCR reaction X 40 µL (total dPCR reaction volume) / 5 µL (DNA input volume) X 200 µL (DNA elution volume). ** Nb of taeniid eggs estimated = Nb of mtDNA copies / 5261 copies (average mtDNA copies per *E. multilocularis* egg after DNA extraction using the DNA Blood & Tissue Kit, Qiagen).

				digital PCR results				
Sample ID	Food item	Country	Parasite species	Conc. [cp/µL] (dPCR reaction)	Valid partitions	Positive partitions	Nb of mtDNA copies*	Nb of taeniid eggs** (raw estimate)
SAL 22028	lettuce	France	*E. multilocularis*	0.793	25277	15	1269	1 (0.24)
SAL 22346	lettuce	Switzerland	*E. multilocularis*	0.106	25429	2	170	1 (0.03)
SAL 22415	lettuce	Denmark	*E. multilocularis*	0.752	25442	14	1203	1 (0.23)
SAL 22417	lettuce	Denmark	*E. multilocularis*	0.381	25461	7	610	1 (0.12)
SAL23944	chard	Germany	*E. multilocularis*	31.470	25445	587	50,352	**10 (9.57)**
FRA 24625	strawberry	Latvia	*E. multilocularis*	0.266	25445	5	426	1 (0.08)
FRA 24629	strawberry	Latvia	*E. multilocularis*	0.215	25440	4	344	1 (0.07)
MYR 24657	blueberry	Latvia	*E. multilocularis*	0.387	25455	7	619	1 (0.12)
MYR 24668	blueberry	Latvia	*E. multilocularis*	0.717	25457	13	1147	1 (0.22)
MYR 24669	blueberry	Latvia	*E. multilocularis*	0.164	25475	3	262	1 (0.05)
FRA 24808	strawberry	Netherlands	*E. multilocularis*	0.958	25454	18	1533	1 (0.29)
MYR 24810	blueberry	Netherlands	*E. multilocularis*	0.159	25460	3	254	1 (0.05)
FRA 24825	strawberry	Denmark	*E. multilocularis*	6.478	25473	124	10,365	**2 (1.97)**
FRA 24866	strawberry	Estonia	*E. multilocularis*	1.888	25459	35	3021	1 (0.57)
FRA 24869	strawberry	Estonia	*E. multilocularis*	2.809	25469	51	4494	1 (0.85)
FRA 24876	strawberry	Estonia	*E. multilocularis*	3.424	25447	64	5478	**2 (1.04)**
MYR 25024	blueberry	Pakistan	*E. multilocularis*	2.551	25462	48	4082	1 (0.78)
MYR 25026	blueberry	Pakistan	*E. multilocularis*	25.380	25447	468	40,608	**8 (7.72)**
MYR 25027	blueberry	Pakistan	*E. multilocularis*	11.340	25465	211	18,144	**4 (3.45)**
MYR 25029	blueberry	Pakistan	*E. multilocularis*	1.993	25327	38	3189	1 (0.61)
MYR 25031	blueberry	Pakistan	*E. multilocularis*	0.157	25441	3	251	1 (0.05)
MYR 25039	blueberry	Pakistan	*E. multilocularis*	0.221	25463	4	353	1 (0.07)
MYR 25041	blueberry	Pakistan	*E. multilocularis*	0.164	25477	3	262	1 (0.05)
SAL 23103	lettuce	Italy (Sardinia)	*E. granulosus s.s.*	0.214	25425	4	342	1 (0.07)
SAL 23518	lettuce	Tunisia	*E. granulosus s.s.*	0.053	25292	1	85	1 (0.02)
SAL 23529	lettuce	Tunisia	*E. granulosus s.s.*	0.106	25428	2	170	1 (0.03)
SAL 23530	lettuce	Tunisia	*E. granulosus s.s.*	0.161	25446	3	258	1 (0.05)
SAL 23567	lettuce	Italy (Campania)	*E. granulosus s.s.*	0.054	25448	1	86	1 (0.02)
SAL 23578	lettuce	Italy (Campania)	*E. granulosus s.s.*	0.108	25402	2	173	1 (0.03)
SAL 23590	lettuce	Italy (Campania)	*E. granulosus s.s.*	0.055	25427	1	88	1 (0.02)
FRA 24199	strawberry	Tunisia	*E. granulosus s.s.*	0.107	25368	2	171	1 (0.03)
FRA 24200	strawberry	Tunisia	*E. granulosus s.s.*	0.107	25391	2	171	1 (0.03)
FRA 24201	strawberry	Tunisia	*E. granulosus s.s.*	0.107	25469	2	171	1 (0.03)
FRA 24203	strawberry	Tunisia	*E. granulosus s.s.*	0.106	25436	2	170	1 (0.03)
FRA 24204	strawberry	Tunisia	*E. granulosus s.s.*	15.970	25428	304	25,552	5 (4.86)
FRA 24205	strawberry	Tunisia	*E. granulosus s.s.*	0.105	25452	2	168	1 (0.03)
FRA 24206	strawberry	Tunisia	*E. granulosus s.s.*	0.270	25457	5	432	1 (0.08)
FRA 24208	strawberry	Tunisia	*E. granulosus s.s.*	0.331	25425	6	530	1 (0.10)
FRA 24213	strawberry	Tunisia	*E. granulosus s.s.*	0.11	25448	2	176	1 (0.03)
FRA 24214	strawberry	Tunisia	*E. granulosus s.s.*	5.582	25424	102	8931	**2 (1.70)**
MYR 24673	blueberry	Latvia	*E. granulosus s.s.*	0.160	25429	3	256	1 (0.05)
FRA 24748	strawberry	Italy (Sardinia)	*E. granulosus s.s.*	8.256	25317	156	13,210	**3 (2.51)**
MYR 25036	blueberry	Pakistan	*E. granulosus s.s.*	3.006	25464	56	4810	1 (0.91)

(*continued on next page*)

Table 5 (*continued*)

Sample ID	Food item	Country	Parasite species	digital PCR results				
				Conc. [cp/µL] (dPCR reaction)	Valid partitions	Positive partitions	Nb of mtDNA copies*	Nb of taeniid eggs** (raw estimate)
MYR 25045	blueberry	Pakistan	*E. granulosus s.s.*	1.732	25390	32	2771	1 (0.53)
SAL 23500	lettuce	Pakistan	*Taenia hydatigena*	0.209	25460	4	334	1 (0.06)
FRA 24763	strawberry	Italy (Sardinia)	*Hydatigera* sp.	0.557	25460	10	891	1 (0.17)
FRA 24764	strawberry	Italy (Sardinia)	*Hydatigera* sp.	1.106	25430	20	1770	1 (0.34)

Table 6
Results of the performance comparison between microscopy and digital PCR, for the estimation of the number of *E. multilocularis* eggs spiked in washing pellets (200 µL) obtained from 300 g of lettuces leaves followed by magnetic DNA extraction. For samples spiked with 63 and 34 eggs, DNA was diluted 1:10 prior to digital PCR; a factor of 10 was applied to the calculation of mtDNA copies. * Nb of mtDNA copies = Conc. [cp/µL] dPCR reaction X 40 µL (total dPCR reaction volume) / 5 µL (DNA input volume) X 100 µL (DNA elution volume). ** Nb of taeniid eggs estimated = Nb of mtDNA copies / 1741 copies (average mtDNA copies per *E. multilocularis* egg after magnetic bead-based DNA extraction).

Nb of *E. multilocularis* egg spiked	Microscopy		Digital PCR				
	Nb of *E. multilocularis* eggs observed by operator 1	Nb of *E. multilocularis* eggs observed by operator 2	Conc. [cp/µL] (dPCR reaction)	Valid partitions	Positive partitions	Nb of mtDNA copies *	Nb estimated of taeniid eggs (difference vs nb eggs spiked) **
63	2	4	10.998	25,432	201	87.986	51 (−12)
34	5	5	5.164	25,428	96	41.316	24 (−10)
21	6	0	30.373	25,354	565	24.298	14 (−7)
17	3	3	21.260	25,383	390	17.008	10 (−7)
15	7	2	27.823	25,286	492	21,826	13 (−2)
8	0	0	6.441	25,360	122	5153	3 (−5)
5	1	0	4.046	25,407	73	3237	2 (−3)
5	0	0	7.906	25,459	147	6325	4 (−1)
5	2	2	7.289	24,877	133	5381	4 (−1)
3	0	0	4.138	25,474	75	3310	2 (−1)
2	1	1	0.480	25,406	9	384	1 (−1)
1	0	0	0.000	25,456	0	0	0 (−1)
1	0	0	1.921	25,428	35	1537	1 (0)
1	0	0	0.545	25,471	10	436	1 (0)
0	0	0	0.054	25,443	1	Not applicable	Not applicable

samples detected, based on the highest operator count) and 93% using real-time PCR (13/14 samples detected). These values decreased to 38% for microscopy (3/8 samples detected) and 88% for real-time PCR (7/8 samples detected) when considering only the eight samples spiked with one to five eggs. Adjusting for most (95.7%) food samples containing five eggs or fewer, the estimated sensitivity of microscopy and real-time PCR under natural contamination levels was 40.0% and 88.5%, respectively.

The correlation between the number of eggs added to the samples and microscopy counts for all samples was moderate ($r = 0.67$, $p < 0.05$). As a low number of eggs was generally observed (maximum of seven in one sample), a lower correlation value was obtained for samples containing more than five eggs ($r = 0.30$) compared to samples with five eggs or fewer ($r = 0.50$). The number of eggs estimated by dPCR was strongly correlated with the number of eggs added to the samples (Pearson's $r = 0.99$, $p < 0.01$). When considering samples with more than five eggs, this correlation remained strong ($r = 0.99$), and was slightly lower but still strong for samples with five eggs or fewer ($r = 0.90$). Correlation plots visually confirmed that dPCR was more strongly correlated and provided a more accurate estimate of egg numbers than microscopy (Fig. 2). For both methods, the estimated counts were consistently lower than the actual number of eggs added to the samples, with mean absolute error values of 10.1 and 3.4 for microscopy and dPCR, respectively. Mean absolute percentage errors were comparable across egg count categories (< 5 vs. > 5 eggs) for each method, ranging between 33 and 35% for dPCR and 81–86% for microscopy.

4. Discussion

To date, only one study has provided an estimation of the number of mtDNA copies present in one taeniid egg (Trachsel et al.,

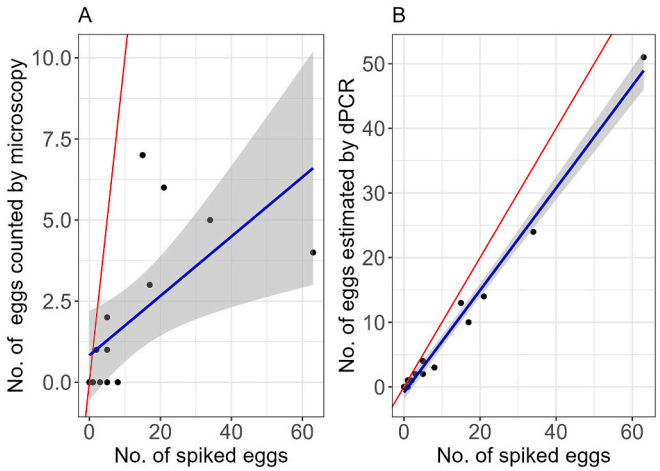

Fig. 2. Linear regression between spiked and estimated numbers of *E. multilocularis* eggs in food pellets using microscopy (A) and digital PCR (dPCR) (B). Blue lines indicate fitted regressions (95% CI in grey), and the red line represents perfect agreement. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2007). It was therefore deemed warranted to re-examine this estimate with the benefit of advanced detection technologies, thereby allowing more precise quantification of taeniid eggs present in food or other environmental samples. The dPCR has been shown to exhibit superior sensitivity, precision and accuracy compared with traditional diagnostic methods, particularly in scenarios involving small parasite populations (i.e. low-intensity infection) (Baltrušis and Höglund, 2023). This technological advance positions dPCR as unmatched among amplification-based methods. In the present study, dPCR was applied to achieve accurate quantification of the two major *Echinococcus* species, as well as the ubiquitous *T. hydatigena,* in food samples.

Using a column-based DNA extraction kit, an estimate of 5261 mtDNA copies (±1253) was obtained for a single *E. multilocularis* egg. Although similar values were expected for *E. granulosus* s.s. and *T. hydatigena*, lower values were obtained (3445 ±1179 and 3203 ±1608, respectively). This difference may be explained by reduced DNA integrity in field-collected fecal samples, as fresh eggs from these two species were not available for testing.

Nevertheless, the highest copy numbers estimated for *E. granulosus* s.s. and *T. hydatigena* eggs were close to the average value obtained for *E. multilocularis*, without exceeding it (except for one *T. hydatigena* egg with 7304 copies), which may suggest that these values reflect more preserved eggs. While one could argue that biological differences between species of the Taeniidae family impact mtDNA copy number, further analyses using fresh samples from species other than *E. multilocularis,* and especially from the *Taenia* genus, would be necessary to clarify this matter. Still, as similar values were obtained among different batches of two species from different genera (i.e. *E. granulosus* s.s. and *T. hydatigena*), this reinforces the hypothesis previously established by Trachsel et al. (Trachsel et al., 2007) that the number of mtDNA copies in a taeniid egg is likely comparable across species of the Taeniidae family.

All of the above supports the use of the estimate obtained here for *E. multilocularis* (5261+/− 1253 copies per egg) for determining the number of taeniid eggs present in contaminated food samples, regardless of the taeniid species involved. If values obtained for *E. granulosus* s.s. or *T. hydatigena* were used, the proportion of food samples contaminated by one to five eggs would not have changed as only one *E. granulosus* s.s. positive sample would shift to the category of more than five eggs. However, the use of a different DNA extraction method (i.e. magnetic bead-based) provided a lower estimate of the mtDNA copy number for *E. multilocularis,* most probably due to its lower efficiency. While further tests are required to confirm these differences, this highlights the need to obtain a reference value that complies with the protocol used for food samples in order to achieve accurate estimation.

The reference value obtained here for *E. multilocularis* eggs should be used with caution because some differences in the estimated number of mtDNA copies were observed within the same batch of eggs, even for fresh viable eggs of *E. multilocularis*. It is hypothesized that this difference is primarily attributable to biological variations in mitochondria-content and cell number among the eggs, rather than to technical factors. Based on our estimation of mtDNA copies per egg and as an oncosphere contains around 54 cells (Swiderski, 1983), we estimate an average of around 95 mitochondria per cell. The value of 5261 copies (± 1253) is lower than the previously obtained value for *T. hydatigena*, estimated at 7100 copies (±1900). This difference may be justified by the absolute estimate obtained by dPCR, which has been shown to be more accurate than real-time PCR, the latter relying on a calibration curve dependent on the calibration of the internal standard. The process of washing and filtration (as in microscopy) may result in the loss of eggs, consequently leading to underestimation regardless of the detection method. In our study, however, as demonstrated by the limit of detection of the washing/filtration method used (Guggisberg et al., 2020; Umhang et al., 2025), this phenomenon is likely to affect only one or two eggs at most. Consequently, it is unlikely to exert a significant influence on the overall estimate of egg numbers by dPCR, regardless of the DNA extraction method used.

The usual food contamination level by *E. multilocularis*, *E. granulosus* s.s. or *T. hydatigena* observed in our study corresponds to one to five eggs in 95.7% of the samples. Such low numbers of eggs may be overlooked by microscopy, which explains the generally lower detection rates in food samples by microscopy compared to molecular methods (Barosi and Umhang, 2024). For instance, the

proportion of lettuce samples contaminated by taeniid eggs collected in local markets in Tunisia was estimated at 2.5% ($n = 40$, one positive sample for *E. granulosus* s.s.) by microscopy versus 14.7% ($n = 75$, nine samples positive for *E. granulosus* s.s., two for *T. hydatigena*, and one for both) by molecular methods (Umhang et al., 2025). The performance comparison conducted in that study demonstrated superior sensitivity of real-time PCR compared to microscopy, particularly in samples containing between one and five eggs, which according to our results, represent the most frequent contamination levels observed in vegetables and berries. Reporting the number of taeniid eggs present in food samples was previously possible only using microscopy. As indicated in our comparison study, such microscopic observation is subject to operator-dependent variations, irrespective of the washing/filtration process performed upstream. Surprisingly, although microscopic observation of taeniid eggs in food samples has been widely reported (Saelens and Gabriël, 2020), quantitative data on taeniid egg counts in contaminated samples are, to our knowledge, only available from two studies. In the first, conducted in Turkey, seven samples ($n = 4$ parsley, $n = 1$ green onion, cucumber, carrot) were found to contain taeniid eggs, with counts ranging from four to 68 per sample. Notably, only one sample contained fewer than five eggs, with a median value of 12 eggs per sample (Kozan et al., 2007). In the second study, conducted in Tunisia, one lettuce and two chard samples contained 41, 56 and 72 taeniid eggs, respectively, with molecular analyses identifying *E. granulosus* s.s. in all three cases (M'Rad et al., 2020). From these microscopic data, one can infer that contamination of vegetable samples generally involves one to several dozen eggs. The estimation obtained in the present study using dPCR is somewhat different, as the vast majority of contaminated food samples (95.7%) contained one to five eggs, with most (83.3%) containing just one. Still, although up to 10 eggs were detected in the most contaminated food sample in our study, higher levels of contamination, such as those reported in these earlier microscopic studies, likely represent a minor proportion of contaminated samples.

The number of mtDNA copies in taeniid eggs detected per food sample is generally very low, with most values corresponding to less than 0.3 eggs. In addition to degradation in the environment and sample washing, the process of DNA extraction may impact the mtDNA recovery. This is reflected in our study by a lower average mtDNA copy number obtained from the same batch of *E. multilocularis* eggs using DNA extraction based on magnetic beads (1741 ± 727) compared to the column-based method (5261 ± 1263).

It is hypothesized that a high level of mtDNA degradation may be associated with egg non-viability. Unfortunately, there is currently no method to estimate the viability of the small number of *Echinococcus* eggs isolated from food samples (Alvarez Rojas et al., 2018; Barosi and Umhang, 2024). In the absence of such a method, contamination rates of food samples by *Echinococcus* eggs should be interpreted with caution in the context of human infection risk. It is generally assumed that ingestion of a single *E. multilocularis* egg may be sufficient to cause infection in humans. However, experimental data show that only half of mice (two of four) orally challenged with 100 eggs were infected, and none with 20 eggs (Federer et al., 2015). We can therefore hypothesize that food samples contaminated with higher numbers of eggs may increase the risk of human infection. According to the dPCR results obtained in this study, however, such cases represent only a small proportion of contaminated food samples. Nevertheless, the risk of human infection from even a few eggs cannot be excluded, particularly in high-risk groups such as immunocompromised patients (Autier et al., 2023).

5. Conclusion

Our study provides the first estimation of taeniid egg numbers in food samples using a molecular approach. Based on an average of 5261 (±1253) mtDNA copies per *E. multilocularis* egg, dPCR revealed that contamination in most food samples (95.7%) was due to only one to five eggs. These findings also highlight the superior sensitivity of molecular methods compared to microscopy and establish a quantitative basis for assessing human exposure to foodborne taeniid infections. However, it should be kept in mind that while dPCR enables accurate estimation of egg numbers, it does not assess egg viability. Future work should therefore focus on viability testing to refine risk assessment.

CRediT authorship contribution statement

Gérald Umhang: Writing – original draft, Validation, Supervision, Project administration, Investigation, Data curation, Conceptualization. **Bastid Vanessa:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Léane Henry:** Writing – review & editing, Supervision, Investigation. **Fanny Bastien:** Writing – review & editing, Resources, Investigation. **Haroon Ahmed:** Writing – review & editing, Resources, Investigation. **Kees van der Ark:** Writing – review & editing, Resources, Investigation. **Rebecca Berg:** Writing – review & editing, Resources, Investigation. **Piero Bonelli:** Writing – review & editing, Resources, Investigation. **Peter Deplazes:** Writing – review & editing, Resources, Investigation. **Gunita Deksne:** Writing – review & editing, Resources, Investigation. **Joke Van der Giessen:** Writing – review & editing, Resources, Investigation. **Pikka Jokelainen:** Writing – review & editing, Resources, Investigation. **Jacek Karamon:** Writing – review & editing, Resources, Investigation. **Selim M'Rad:** Writing – review & editing, Resources, Investigation. **Pavlo Maksimov:** Writing – review & editing, Resources, Investigation. **Myriam Oudni-M'Rad:** Writing – review & editing, Resources, Investigation. **Paola Pepe:** Writing – review & editing, Resources, Investigation. **Laura Rinaldi:** Writing – review & editing, Resources, Investigation. **Małgorzata Samorek-Pierog:** Writing – review & editing, Resources, Investigation. **Cinzia Santucciu:** Writing – review & editing, Resources, Investigation. **Urmas Saarma:** Writing – review & editing, Resources, Investigation. **Adriano Casulli:** Writing – review & editing, Project administration, Funding acquisition. **Panagiota Ligda:** Writing – review & editing, Resources, Investigation. **Smaragda Sotiraki:** Writing – review & editing, Resources, Investigation. **Laure Bournez:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Sandrine Leselier:** Writing – review & editing, Resources. **Roxanne Barosi:** Writing – review & editing, Supervision, Resources, Investigation, Data curation. **Franck Boué:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Carine Peytavin de Garam:** Writing – review & editing, Validation,

Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics statement

Ethical agreement was obtained from the French ethical committee for animal experimentation and by the French "Ministère de l'Enseignement Supérieur et de la Recherche" on 10th December 2021 (APAFIS #33541-202110081648536 v11).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Umhang Gerald reports financial support was provided by European Union. Gerald Umhang reports financial support was provided by Partnership for Research and Innovation in the Mediterranean area. Urams Saarma reports financial support was provided by Estonian Ministry of Education and Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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