

Preliminary evaluation of different methods to detect and quantify *Taenia* eggs in sludge and water samples: A spiking experiment to assess recovery efficiency

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ABSTRACT

An improved understanding of the environmental transmission of *Taenia* spp. is key to control of the parasite. Methods to detect and quantify *Taenia* eggs in different environmental matrices, including sludge and water, currently lack performance validation with regard to the recovery efficiency and process ease of use. Therefore, this study aimed to assess the recovery efficiency and process duration of commonly used methods for the detection of *Taenia* eggs in sludge and water samples. Ten detection methods for *Taenia* spp. eggs were selected from a systematic review. Sludge and water samples were spiked with a high dose of *Taenia saginata* eggs, i.e., around 200 eggs/g sludge and 50 eggs/ml water, and were tested using five methods each. The two methods with the highest egg recovery efficiencies were selected per matrix for assessment with a lower spiking dose, i.e., 4 eggs/g sludge and 1 egg/ml water. Each time five replicates were used. Recovery efficiency was defined as the proportion of the number of eggs recovered to the total number of eggs spiked. Using the high spiking dose, all samples tested positive for all the methods. The mean egg recovery efficiency varied from 4% to 69% for sludge samples and from 3% to 68% for water samples. Using the lower spiking dose, one of the methods performed on sludge samples was able to detect all replicates, whereas only one replicate was positive using the other method. For water, all low dose samples tested positive using both methods. In conclusion, most methods performed inadequately in recovering *Taenia* eggs from sludge and water, with half of the methods performed on the high dose samples having a mean egg recovery efficiency of approximately 10% or less. The assessed recovery methods were generally time-consuming and labourious. A more thorough validation of existing recovery methods and improvement of method protocols to increase recovery efficiency is thus urgently needed.

1. Introduction

For *Taenia solium*, *Taenia saginata* and *Taenia asiatica*, which are neglected foodborne zoonoses with a major public health and economic impact, environmental egg contamination is crucial in disease transmission (Alvarez Rojas et al., 2018). Tapeworm carriers

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may excrete tens of thousands of eggs daily, potentially extensively contaminating the environment via open-air defecation or, for example, via sewage and water purification plant effluent. Accordingly, a high environmental egg load is putting animals and people at risk of infection (Jansen et al., 2021; Saelens et al., 2022). Soil, water, and food/feed are the most important potential environmental transmission matrices. Determination of the environmental contamination load of these matrices can aid in the evaluation of control interventions and determination of at-risk areas in need for interventions and is, as such, key to sustained control and ultimately elimination of the parasite (Saelens et al., 2022; Steinbaum et al., 2017). Although a large variety of methods have been used to detect and quantify *Taenia* eggs in different environmental matrices, these methods are generally not validated (Alvarez Rojas et al., 2018; Saelens et al., 2022). Validated detection methods are necessary for accurate quantitative assessment of environmental contamination with *Taenia* spp. eggs (Zendejas-Heredia et al., 2021). A first step in determining the contamination load of an environmental sample, before actual microscopical or molecular detection, is the processing of the sample for egg recovery (isolation and concentration), which highly influences the performance of the subsequent detection method (Frey et al., 2019). Many egg recovery methods and protocols have been applied to recover eggs from various environmental matrices. However, appropriate performance validation and determination is mostly lacking. This study therefore aims to determine the recovery efficiency and process ease of use of ten commonly used methods in sludge and water samples that were selected from a previously conducted systematic review by Saelens et al. (2022).

2. Material and methods

2.1. Source of sludge, water and *Taenia saginata* eggs

Active sludge and water (effluent) samples were collected from Flemish mechanical and biological system wastewater treatment plants that applied an activated sludge system for biological purification, and stored at 4 °C until spiking. Active sludge refers to suspended aerobic sludge that contains a mixture of bacteria capable of biodegrading organic matter under aerobic conditions. Three

Table 1

Overview of the technical details of the ten methods used, including the related recovery efficiency, duration, and number of steps.

Method description	Matrix	Mean recovery efficiency high dose (%)	Total time to recovery	Time of labour	Number of steps to result	Reference
Method 1: dilution (0.05% Tween 80), filtration (100 µm mesh), sedimentation (15 s), centrifugation (1000 g for 5 min and 1000 g for 15 min), and sucrose flotation (s.g. 1.27)	Sludge	4	3 h35'	1 h15'	8	Matsudo et al., 2003
Method 2: washing (1% Tween 80 in phosphate-buffered saline), filtration (4mm ² mesh), zinc sulfate flotation (s.g. 1.18), and centrifugation (1000 g for 5 min (2×) and 1000 g for 15 min)	Sludge	6	31 h28'	1 h05'	18	Huerta et al., 2008
Method 3: washing (1% Tween 80 in phosphate-buffered saline), filtration (4mm ² mesh), centrifugation (300 g for 3 min, 838 g for 10 min and 425 g for 2 min), and formalin-ether sedimentation	Sludge	69	27 h20'	55'	15	Huerta et al., 2008
Method 4: dilution (distilled water), filtration (500 µm mesh), sedimentation (30 min), saturated salt flotation (s.g. 1.20), and centrifugation (2000 rpm for 5 min)	Sludge	12	3 h45'	1 h10'	7	Opara and Udoidung, 2003
Method 5: filtration (250–300 µm mesh), Sheather's sugar flotation (s.g. 1.30), and centrifugation (3000 rpm for 10 min)	Sludge	33	2 h15'	2 h05'	6	Shathele and el Hassan, 2009
Method 6: filtration (250–300 µm mesh), Sheather's sugar flotation (s.g. 1.30), and centrifugation (3000 rpm for 10 min)	Water	3	2 h15'	2 h05'	6	Shathele and el Hassan, 2009
Method 7: sedimentation (2 h) and centrifugation (1500 rpm for 10 min)	Water	68	2 h55'	40'	4	Wongworapat et al., 2001
Method 8: modified Bailenger technique based on sedimentation (1–2 h), ethyl acetate, zinc sulfate flotation (s.g. 1.18), and centrifugation (1000 g for 15 min (2×))	Water	18	3 h25'	55'	13	Madera et al., 2002
Method 9: filtration (160 µm mesh) sedimentation (2× overnight), magnesium sulfate flotation (s.g. not specified), and centrifugation (400 g for 3–5 min (2×), 480 g for 3 min (2×) and 660 g for 3 min)	Water	5	1 h55'	1 h15'	6	Verbyla et al., 2013
Method 10: sedimentation (overnight in sodium hydroxide), saturated sodium nitrate flotation for large-volume sediment samples (s.g. 1.18–1.20), and centrifugation (3000 rpm for 10 min and 1000 rpm for 5 min)	Water	56	16 h50'	40'	6–10	Noda et al., 2009

s.g. = specific gravity.

batches of *Taenia saginata* eggs were obtained by manually extracting eggs from gravid proglottids acquired from commercial laboratories. The eggs were initially assessed for integrity by microscopical examination; only proglottids containing intact eggs were selected. Three homogenous egg mixtures were prepared by washing the eggs in phosphate-buffered saline with added antibiotic solution, containing 100 IU/ml penicillin and 100 µg/ml streptomycin. The mean egg concentration of each mixture was microscopically determined at 100× magnification of 50 µl aliquots, with five replicates for each batch. The average concentrations of the three mixtures were estimated at 50.000 eggs/ml, 26.400 eggs/ml, and 41.000 eggs/ml, which were stored for one to three months at 4 °C until spiking.

2.2. Spiking of sludge and water samples

Initially, the sludge and water samples were screened to confirm their negative status with recovery protocols described by (Santos et al., 2004) and Guggisberg et al. (2020), respectively. The sludge and water samples were subsequently spiked with a high dose of *T. saginata* eggs, extracted from one of the three mixtures with pre-defined concentrations. Approximately 200 eggs/g and 50 eggs/ml were pipetted onto the sludge (50 g) and water (250 ml) samples, respectively, and mixed thoroughly. Next, eggs were recovered from all spiked samples using ten methods (see section 2.3.). For each matrix, the two methods with the highest recovery efficiencies were selected for further validation using a lower spiking dose, i.e., 4 eggs/g sludge and 1 egg/ml water. Importantly, while these lower spiking doses are hereafter referred to as 'low', they still represent relatively high doses, considering the weight and volume of the sludge and water samples. For each method/dose combination, the spiking experiment was performed in five replicates.

2.3. Recovery of eggs from sludge and water samples

Eggs were recovered from all replicate sludge and water samples spiked with the high doses using ten methods in total, five for each matrix. These methods were selected from a previously conducted systematic review (Saelens et al., 2022). Method 1 to 5 was performed on sludge samples and method 6 to 10 on water samples (Table 1). The duration of each step within all method protocols was tracked. Following the implementation of each method, eggs recovered from aliquot samples were microscopically enumerated at 100× magnification. Based on the number of eggs in the aliquot samples, the mean recovery efficiency was calculated per method. Thereafter, for each matrix, the two methods with the highest recovery efficiencies were identified and performed again on sludge and water samples spiked with the low doses. The mean recovery efficiency was once more calculated per method.

2.4. Data analysis

Recovery experiment results were descriptively analysed using RStudio version 4.0.2 (R Core Team, 2021). Recovery efficiency was calculated by dividing the final number of eggs recovered by the initial number of eggs spiked. The 95% bootstrapped confidence

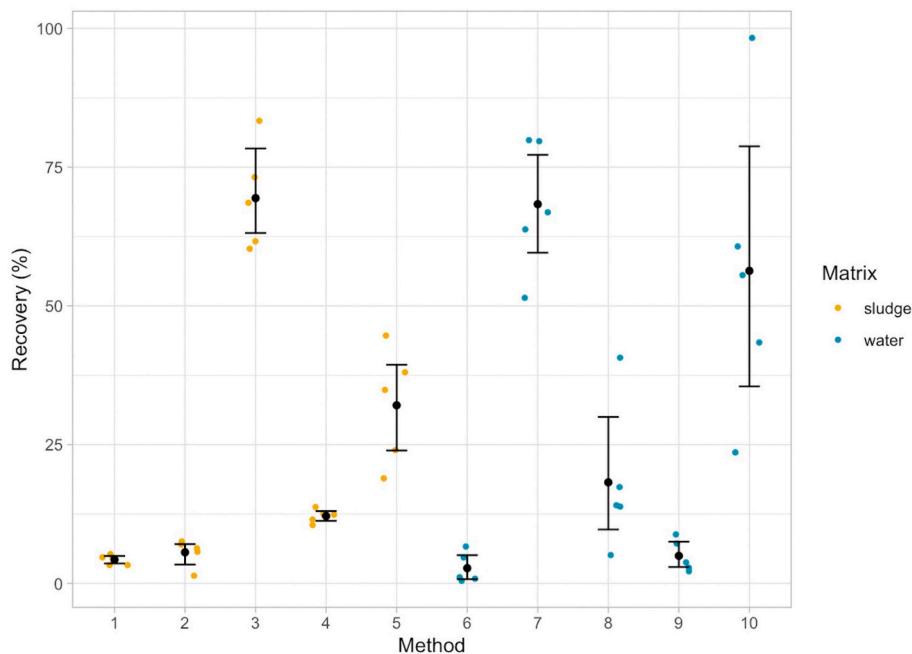


Fig. 1. Egg recovery efficiency of the ten methods performed on sludge and water samples spiked with the high doses, i.e., around 200 eggs/g sludge (method 1–5) and 50 eggs/ml water (method 6–10). The error bars indicate the 95% confidence interval.

intervals for the recovery efficiency were determined using the accelerated bias-corrected method (Lisi, 2022).

3. Results

3.1. Recovery efficiency of high spiking doses

Using the high spiking doses, eggs were recovered from all samples tested by each of the methods. The recovery efficiency results for all methods performed on samples spiked with the high doses are provided in Table 1. Overall, half of the methods had a mean recovery efficiency of approximately 10% or less. The mean egg recovery efficiency varied from 4% (method 1) to 69% (method 3) for sludge samples and from 3% (method 6) to 68% (method 7) for water samples (Table 1). However, for half of the methods there was considerable variation in recovery efficiency between replicate measurements ($n = 5$). Especially for method 10, a large confidence interval could be observed (Fig. 1). Because method 3 (69%) and 5 (33%) yielded the highest recovery efficiencies for sludge samples spiked with the high dose, these were subsequently applied on sludge samples spiked with the low dose. Likewise, for water samples, method 7 (68%) and 10 (56%) were applied on the samples spiked with the low dose.

3.2. Recovery efficiency of low spiking doses

For sludge, method 3 was able to recover eggs from all five low dose samples, but eggs could only be recovered from one sample using method 5. Eggs were recovered from all water samples spiked with the low dose ($n = 5$) using both method 7 and 10. Since very high variation in recovery efficiency between replicate measurements ($n = 5$) was observed for method 3, 7 and 10, and multiple measurements had a recovery efficiency of greater than 100%, the mean recovery efficiency results for the low spiking doses were considered unreliable.

3.3. Ease of use: method duration (time to recovery) and number of steps in sample handling/preparation (steps to recovery)

The total method duration varied between 2 h15' (method 5) and 31 h28' (method 2) for sludge samples and between 1 h55' (method 9) and 16 h50' (method 10) for water samples (Table 1). The duration of actual labour varied between 55' (method 3) and 2 h05' (method 5) for sludge samples and between 40' (method 7 and 10) and 2 h05' (method 6) for water samples. Generally, the number of steps required to recover eggs ranged from 4 to 18, with most methods requiring ten steps or less.

4. Discussion

This preliminary study showed that the evaluated recovery methods are currently insufficient to determine environmental *Taenia* egg contamination, as major egg losses occur during processing. Despite various recovery protocols being described in the literature, they generally lack performance validation. A recent systematic review revealed that out of 57 records assessing egg presence in soil and water, only two assessed the recovery efficiency of the applied protocol, which were reported to be low (Saelens et al., 2022). A recovery efficiency of 6.7–20.9% was described following a formalin-ether sedimentation and zinc sulfate flotation technique applied on different types of sewage sludge (Satchwell, 1986), while a recovery efficiency of 2.5% was described for silt samples on which a Sheather's sucrose flotation-centrifugation technique was applied (Scandrett and Gajadhar, 2004). Higher recovery efficiencies were reported in the present study; method 3 (69%) and 7 (68%) were the most efficient at recovering eggs from sludge and water samples spiked with the high doses, respectively. However, it should be considered that further loss of eggs or DNA will most likely occur during the subsequent DNA extraction and detection procedures (Schrader et al., 2012).

On top of the unsatisfactory recovery efficiencies they yielded, the assessed protocols were often time-consuming and labourious, challenging their implementation in a field setting. Surprisingly, the actual time of labour needed to perform the slowest recovery method was still lower than the actual time of labour needed to perform the fastest recovery method. Also, a great variety in the number of steps was observed between methods, which ranged between 4 and 18 steps. The duration and number of steps should of course be considered in the organisation of the lab work.

There are some limitations of the recovery efficiency experiments that should be addressed. First, baseline contamination of the sludge and water samples was assessed with methods yielding suboptimal recovery efficiencies. Given that most wastewater treatment plants fail to fully eliminate *Taenia* spp. eggs, it is possible that false-negative samples were present (Jansen et al., 2021). As such, baseline contamination of the environmental samples with *T. saginata* eggs or other *Taenia* spp. eggs, which are microscopically indistinguishable, could have resulted in an overestimation of the recovery efficiencies of the assessed methods (Jimenez et al., 2010). Egg contamination at baseline might also explain the mean recovery efficiency of greater than 100% that was observed when applying method 10 on water samples spiked with the low dose. Nevertheless, this could also result from inaccurate spiking because the determination of the concentrations of the egg mixtures was based on aliquots. Accordingly, the concentrations of the entire egg mixtures were estimations, bearing a certain level of uncertainty. It is thus possible that the actual number of eggs in the high and low spiking doses was lower or higher than assumed. Also, since the microscopical enumeration of eggs after recovery was performed on relatively small aliquots, small measurement errors might have a big impact on recovery efficiency determination. Alternatively, because these have a higher sensitivity compared to microscopical examination, molecular tools could be used to determine the recovery efficiency. The latter, however, detects egg DNA instead of eggs (Mayta et al., 2008). Another limitation is that, for certain methods, there was considerable variation in recovery efficiencies between replicates, which challenges the detection of significant

differences between different protocols (Steinbaum et al., 2017). This variation might be related to spiking variability, pipetting errors, the lack of sensitive detection and enumeration, or imperfect homogenization of the spiked samples. The latter implies that the aliquot might not be representative for the entire sample, thus, yielding unreliable recovery efficiencies. Furthermore, the variation in recovery efficiencies might be due to differences in egg quality between batches. Due to the variable accessibility of proglottids during the study period, replicate samples were spiked with different egg batches. Thus, spiking samples with a batch of eggs with lower integrity and lower viability could have led to rapid disintegration of eggs during the subsequent recovery procedures, eventually yielding lower recovery efficiencies. Even though an attempt was made to select intact eggs to obtain comparable batches, viability assessment was not performed.

The evaluated recovery methods in this study involved a combination of protocol steps such as washing, centrifugation, flotation, filtration, and sedimentation; though, each method applied different centrifugation settings, sedimentation times, and flotation solutions. This lack of standardization complicates the comparison of results between studies. Ultimately, the recovery efficiency of a protocol depends on the differing technical settings of each step in the protocol (Saelens et al., 2022). The flotation solution appears to be particularly important because *Taenia* eggs require a flotation solution with a specific gravity of at least 1.27 (Maya et al., 2006). Unfortunately, the specific gravity was lower than 1.27 for some methods reported in the systematic review or not specified at all (Saelens et al., 2022). On the other hand, flotation solutions with high specific gravity often interfere with the separation of eggs from debris. The latter complicates the analysis of environmental samples further as it is difficult to separate from *Taenia* eggs and challenges microscopical egg detection by reducing the likelihood of detecting eggs (Scandrett and Gajadhar, 2004). Overall, while multiple isolating and concentrating steps might result in increasingly purified eggs, it also increases the risk of losing eggs (Frey et al., 2019). By minimizing processing steps, environmental PCR inhibitors such as debris and humic/fulvic acids will be removed less effectively, which might interfere with subsequent DNA extraction and detection (Schrader et al., 2012).

Since control and elimination of *Taenia* spp. is challenged by environmental egg dispersal, elaboration on the modes of egg transmission in the environment is needed. However, application of the currently unvalidated recovery methods in the field would lead to an underestimation of the egg burden, especially considering the diluted and heterogenous distribution of parasite eggs in environmental matrices such as sludge and water. Because misdiagnosis and performance variation are more likely to occur in samples with a low number of eggs, a highly effective recovery/detection protocol is warranted (Maganira et al., 2019). This study further acknowledges the complexity of developing a satisfactory recovery method and protocol. Ideally, a recovery method for high-throughput processing of environmental samples in low-resource field settings allows for fast and reliable egg enumeration, species identification, and viability determination (Steinbaum et al., 2017). Because such a method is currently unavailable, profound analyses are required to determine the combination of protocol steps and their technical settings that will yield an optimal recovery efficiency, while minimally impacting viability assessment. Currently, the influence of the egg recovery methods on viability remains undressed, yet is crucial information to accurately estimate infection risk as the presence of eggs does not imply viability (Alvarez Rojas et al., 2018).

Altogether, further research is necessary to validate existing protocols and develop improved protocols, which should include determination of the actual recovery efficiency for different environmental matrices. Both experimentally spiked samples as well as field samples should be assessed. In the end, the recovery efficiency should be considered when determining which detection protocol to apply as well as during the interpretation of the test results.

5. Conclusions

Most methods were time-consuming, labourious, and performed inadequately in recovering *Taenia* eggs from sludge and water, with half of the methods performed on the high dose samples having a mean egg recovery efficiency of approximately 10% or less. Methods to recover eggs from environmental matrices should therefore be standardized and validated more thoroughly, and the development of harmonized methodologies is recommended to allow a better comparison between studies. Performance validation should not only consider the recovery efficiency of a recovery method and related protocol, but also the impact of the latter on viability assessment.

Declaration of Competing Interest

The authors 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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