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Comparison of the effectiveness of various parasitological methods in detecting nematode eggs in different types of soil

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Abstract

Introduction and Objective. Natural fertilizers, sewage sludge, digestates, as well as organic fertilizers produced on their basis, can become a source of parasitological contamination of cultivated land. High concentration of invasive forms of parasites in the soil may pose a threat to human and animal health. Therefore, it is necessary to control the hygienic condition of fertilizers and fertilized soils with particular emphasis on parasites. The aim of the study was to compare the effectiveness of methods commonly used for parasitological examination of soil with own methods which were used to develop the standards.

Materials and method. The study was carried out using samples of sandy soil (SS), horticultural mix soil (HS) and peat-based substrate (PS). Each sample was spiked with 100 dyed *Ascaris suum* eggs and examined with the use of 6 methods: Vasilkova, Dada, Quinn, and 3 methods according to the Polish Standards (PN-19000, PN- 19005, PN-19006). For each variant, 8 repetitions were made.

Results. The largest number of *A. suum* eggs were found with PN-19006 (mean number of detected eggs was 21.25, 46.50, 23.00 for HS, SS, PS, respectively. Slightly lower results were obtained using PN-19005 – the mean number eggs was 21.25, 36.00, 16.75, respectively. On the other hand, the mean number of *A. suum* eggs found with the Dada method was about 2–3 times lower than with the PN-19006 – 15.75, 22.50, 6.50 for HS, SS, PS soil, respectively. Other methods were much less effective.

Conclusions. PN-19006 method turned out to be the most effective in detecting *A. suum* eggs. This method can be used for parasitological examination of soils and can be the basis for developing a system of methods dedicated to testing different types of soils for the presence of nematode eggs.

Key words

parasite eggs, soil analysis, parasitological methods, *Ascaris suum*

INTRODUCTION AND OBJECTIVE

Intensive farming requires the use of large amounts of fertilizer on croplands in order to maintain sufficient soil fertility, and to achieve the optimum yield in given conditions. These are primarily mineral fertilizers that supply plants with nitrogen, phosphorus, potassium, boron, sulphur as well as calcium and magnesium. In addition to the content of nutrients, the presence of organic matter in the soil – humus – has a huge impact on the production function of the soil. It determines the physical, physicochemical, chemical and biological properties of the soil, affects among others its structure, aeration and water retention. It makes mineral compounds less susceptible to leaching and slower to penetrate into deeper layers of the soil, beyond the root zone of plants. A source of both nutrients and organic matter can be organic, organic-mineral and natural fertilizers. Organic fertilizers are produced from organic waste, such as waste generated in the process of animal husbandry (manure, slurry), waste from the agricultural and food industries (including slaughterhouses and meat processing plants), municipal organic waste, municipal sewage sludge and digestates from biogas plants. Organic waste is a valuable raw material for the production of fertilizers, but on the other hand, it can be a source of heavy metals, viral, bacterial and parasitic pathogens. Despite the controls applied, such dangerous factors can get into the soil together with fertilizers. In some cases, e.g. when using natural fertilizers (e.g.

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spreading manure directly to the field), or direct application of stabilized sewage sludge or digestates for fertilization, such control is often insufficient or completely absent.

In this way, pathogenic microorganisms and eggs of intestinal parasites can enter the fertilized soil. It should be emphasized that especially the parasites are characterized by significant resistance to environmental conditions – e.g. roundworm eggs in the soil can survive for a very long period of time – even up to 10 years [1, 2]. This phenomenon can lead to the accumulation of invasive eggs of parasites in soils and pose a threat to human and animal health. Therefore, the need to control the hygienic condition of soils, especially those used for the production of food of plant origin intended for direct consumption.

There are many methods used for the parasitological examination of soil. The most commonly used in Poland are: the method according to Vasilkova and Gefter [3], the method according to Dada [4], the Polish Standard PN-Z-19000–4 [5], and the method according to Quinn [6] modified by Gundłach [7]. These methods were developed many years ago and have not been fully validated by the authors.

The Department of Parasitology and Invasive Diseases of the National Veterinary Research Institute in Pulawy, Poland (PIWet) conducts studies on the hygienic condition (in particular – parasitological contamination) of soils for which organic fertilizers were applied. For many years, PIWet has also been conducting parasitological examination of organic fertilizers and sewage sludge, for which it was appointed by the Regulation of the Minister of Agriculture and Rural Development. PIWet uses its own methods because the methods commonly used for this purpose have proved to be insufficiently effective. Own methods have been validated, and on their basis the relevant standards (PN-Z-19005:2018–10 and PN-Z-19006:2023–4) [8, 9] have been developed and issued by the Polish Committee for Standardization.

The PN-Z-19005:2018–10 method [8] was created for the study of sewage sludge dewatered with the use of flocculants (polyelectrolytes), substances which reduce the effectiveness of detection of parasite eggs by traditional flotation methods [10]. This effect of polyelectrolytes in the own method was largely eliminated by long-term breaking of the floccules during mixing in the Tween 20 solution, and a large dilution (1:16) of the sample with the flotation fluid. [11]. The second method developed to test organic fertilizers that do not contain sewage sludge was created by modifying the first method. Due to the lack of flocculants in the tested samples and the lack of their negative impact on the structure of the sample, a smaller 'thinning' of the analytical sample with the flotation fluid (1:4) was used. This allowed an increase in the mass of the analytical sample and, as a result, to increase the sensitivity of the method [9].

These methods are increasingly used by other diagnostic laboratories not only for sewage sludge and organic fertilizers examination, but also to test soil. However, their effectiveness in parasitological examination of soil has not been determined in the validation process.

OBJECTIVE

The aim of the study was to evaluate the effectiveness of methods commonly used for the parasitological examination of soil, and to compare them with our own methods. According to own experience and literature data [12], the factor that makes this examination particularly difficult is the high content of organic matter. Therefore, it was decided to carry out comparative studies on soil samples with different organic matter content. The samples were spiked with most common parasite eggs found in soils – *Ascaris suum.*

MATERIALS AND METHOD

Study design and sample preparation. The experiments were carried out using samples of 3 different types of soil, significantly differing in structure and organic matter content: horticultural mix soil (HS) (pH 5.5 to 6.0), sandy soil (SS) (pH 5 to 5.5), and peat-based substrate PS (pH 3.5 to 4.5). The sandy soil was obtained from agricultural land. Other types of soil material were purchased from a market. The purchased soil was packed in 10 kg bags. Before using for the study, each type of soil was parasitologically tested 3 times using the PN-Z-19006:2023-4 method [9]. No intestinal parasite eggs were found in any of the samples.

Each of the analytical soil samples (of method-dependent weight) were spiked with 100 eggs of *Ascaris suum,* obtained from the uteri of mature female nematodes isolated from the intestines of slaughtered pigs naturally infected with these parasites. Eggs were preserved in 1% formalin solution. They were then subjected to ultrasound for 15 minutes in order to break up the clusters of adjacent eggs. The obtained eggs suspension was stirred on a magnetic stirrer for 10 minutes. In order to distinguish them from the eggs of nematodes that could potentially occur in the tested soil samples, *A. suum* eggs prepared for the experiment were stained with a 2% eosin solution (20 ml of a 2% eosin solution was added to the eggs suspended in 5 ml of 1% formalin solution), and left for 24 h at room temperature. Prior to spiking, the dyed egg suspension was stirred on a magnetic stirrer.

Then, using an automatic pipette, 100 µl of the suspension were taken and placed on a glass slide, creating a long, narrow drop. Using a stereoscopic microscope (magnification 40–100x) the eggs on the slide were counted. Stained eggs (100 eggs) counted on glass slides were rinsed with a small amount of water from the glass slide to an analytical sample of soil with the weight resulting from the method used. Using a stereoscopic microscope, the effectiveness of rinsing the eggs from the slide was checked. Soil samples spiked in this way, containing 100 dyed eggs of *A. suum*, were intensively stirred for 15 min with a mechanical stirrer. Then, proceeded according to the protocol of each method, and for each variant (method and type of soil), 8 repetitions were performed.

Spindler's method modified by Vasilkova and Gefter (Vasilkova) [3]. Twenty ml of a 5% NaOH solution was added to a 10 g soil sample and allowed to stand for 1 h, stirring several times. The sample was then centrifuged for 2 min. at 2,500 x g. After centrifugation, the supernatant was decanted and the sediment layered with 50 ml of saturated $NaNO_3$. The sample was centrifuged 5 times for 2 min at 2500 x g. Each time after centrifugation, approx. 1 ml of the surface layer of the supernatant was collected with a disposable pipette, poured into a beaker with a small amount of water and filtered through polycarbonate filters (alternatively to paper filters). The filters were placed on glass slides and examined under a microscope (magnification 100–200x)

Method according to Dada (Dada) [4]. To a 10 g soil sample, previously sieved through a sieve with a diameter of 1–2 mm mesh containing 100 eggs of *A. suum*, 50 ml of a solution of 0.1 % Tween 80 (1 liter H_2O + 1 ml Tween 80) was added and mixed with a glass rod. The sample was transferred to centrifuge tubes. The level of soil suspension in the tubes was marked with a waterproof marker and then centrifuged for 10 min at 1,500 × g. After centrifugation, the supernatant was removed and to the pellet was added a saturated solution of $ZnSO_4$ (727.5g $ZnSO_4$ in 1 L H_2O) to the volume marked on the tube. The sample was centrifuged again for 10 min. at 1,500 × g. After centrifugation, the tubes were filled with $ZnSO_4$ solution until a convex meniscus was obtained and a coverslip was placed on top of the tube. After 15 min, the coverslip was transferred to a microscope slide and the preparations examined under a microscope (magnification 100–200x).

Quinn's method modified by Gundłach (Quinn) [6, 7]. 50 ml of 0.0025% Tween 80 solution was added to a 50 g soil sample. The sample was homogenized for 1 min stirring with a glass rod. The suspension was filtered through metal sieves with a mesh size of 200 µm. The sample was centrifuged for 10 min at 2,600 x g. After centrifugation, the supernatant was decanted, and the sediment was refilled with Tween 80 solution with a volume of solution similar to the volume of the remaining sediment. The sample was homogenized and centrifuged for 10 min at 2,600 x g. After centrifugation, the supernatant was decanted and the sediment flooded with a saturated NaCl solution. The sample was homogenized for 1 min and centrifuged again for 10 min at 2,600 x g. After centrifugation, the centrifuge tube was refilled with saturated NaCl solution until a convex meniscus was obtained. The tube was covered with a coverslip and then left for 30 min. After this time, the coverslip was transferred to a microscope slide and the preparation was examined under a microscope (magnification 100–200x).

Standardized method PN-Z-19000–4 (PN-19000) [5]. One hundred ml of 5% NaOH solution was added to 100 g soil sample. The suspension was thoroughly mixed and left at room temperature for 1 h. The the sample was then stirred on a magnetic stirrer for 10 min. The soil suspension was transferred to 100 ml centrifuge tubes; before centrifugation, the level of soil suspension was marked on each tube with a waterproof marker. The tubes were centrifuged for 3 min at 780 x g. After that, the supernatant was discarded and the sediment was underlayered with a saturated solution of $NaNO_3$ to the marked level. The suspension was thoroughly mixed and centrifuged again for 3 min at 780 x g. After centrifugation, the surface layer of the liquid (about 2 ml) was collected into a beaker with about 10 ml of water, and the remaining part of the sediment again underlayered with $NaNO_3$ solution to the marked level. The whole solution was mixed and centrifuged 3 min at 780 x g. The resulting suspension was filtered through membrane filters. Four drops of glycerin were applied to the filter and spread with a probe over the entire surface of the filter. The filters were examined under a microscope (magnification 100–200x).

Standardized method PN-Z-19005:2018–10 (PN-19005) [8]. One thousand six hundred ml of a 0.0025% Tween 20 solution was added to 50 g soil sample and mixed with a mechanical mixer for 4 h. The mixed sample was filtered through a 200 µm sieve. The filtrate was poured into centrifuge tubes – at least 4 tubes with a capacity of 400 ml. The tubes were centrifuged for 10 min at 2,500 g and the supernatant removed. The precipitate remaining in the tubes was transferred to a 2 L beaker, 750 ml of a saturated $NaNO_3$ solution was added and the suspension thoroughly mixed. After that, the mixed suspension was centrifuged for 10 min at 2,500 g. After centrifugation, the supernatant – about ½ of the content of each test tube – was poured into a 5 L beaker. The beaker was filled with water to a volume of 5 L and left for about 1.5 h for sedimentation. After that, the supernatant was carefully poured off, leaving about 200 ml of liquid (containing about 10 ml of sediment) in the beaker. The suspension was filtered through polycarbonate filters using a vacuum filtration set. The filters were transferred with tweezers to slides. Four drops of glycerol were applied to each filter and it was thoroughly distributed over the entire surface of the filter. The filters were examined under a microscope (magnification 100–200x).

Standardized method PN-Z-19006:2023-4 (PN-19006) [9]. Four hundred ml of 0.0025% Tween 20 solution was added to a 100 g soil sample and mixed for 1 min on a magnetic stirrer or with a spatula. The suspension was filtered through a sieve with a diameter of 200 µm. The entire obtained filtrate was poured into at least 4 centrifuge tubes (capacity 100 ml) – the level of the sample in the tube was marked with a waterproof marker. The samples were centrifuged for 10 min at 2,500 × g. After centrifugation, the supernatant was removed, and the sediment remaining in the tubes was flooded (up to the volume marked on the tubes) with 0.0025% Tween 20 solution, and mixed well with a spatula. The samples were then centrifuged again for 10 min at 2,500 × g. After centrifugation, the supernatant was removed, and the sediment remaining in the tubes supplemented to the volume marked on the tubes with a saturated solution of $NaNO_3$ and thoroughly stirred with a spatula to render the suspension homogeneous. The samples were centrifuged once again for 10 min at 2,500 × g. After centrifugation, the supernatant – about 2/3 of the content of each tube – was carefully poured into a beaker with a capacity of 1,000 ml, so that about 1/3 of the liquid remained in the tube with the sediment. The beaker was filled with water to a capacity of 1,000 ml and the sample sedimented, leaving it for 1 hour. After sedimentation was finished, the supernatant – about 2/3 of the contents of each tube – was carefully poured out, leaving about 1/3 of the liquid with the sediment in the beaker. The beaker was refilled with water to a volume of 1,000 ml and sedimented again for 1 hour. After sedimentation, the supernatant was carefully poured off, leaving about 200 ml of liquid with sediment in the beaker. The suspension was filtered through polycarbonate filters using a vacuum filtration set. The filters were transferred with tweezers to slides. Four drops of glycerol were applied to each filter and it was thoroughly distributed over the entire surface of the filter. The filters were examined under a microscope (magnification 100–200x).

Statistical analysis

A.suum eggs detected in individual analytical samples were counted. The mean number of detected eggs was calculated for each method. Since the number of eggs added to each analytical sample was equal (100), the mean number of

detected eggs was equal to the egg recovery rate expressed in %. The effectiveness of individual methods was compared, taking into account the mean recovery of eggs and the weight of the analytical sample, and assuming these parameters for the method with the highest recovery rate as 100%. Calculations were made according to the formula:

$$\text{Relative efficiency of the method [\%]} = = \frac{m}{M} * \frac{o}{O} * 100$$

m – mass [g] of the analytical sample in the tested method
M – mass [g] of the analytical sample of the method with the highest egg recovery rate
o – recovery of eggs from the analytical sample of the tested method [%]
O – egg recovery from the analytical sample of the method with the highest egg recovery rate [%].

The significance of differences in the mean numbers of detected eggs from different types of soil tested with different methods was determined using the STATISTICA 7.1 programme (StatSoft Polska). The significance level was α=0.05. The Lilliefors and Shapiro-Wilk tests were used to test the normality of the trait distribution, and the Levene test and the Brown-Forsyth test to test the hypothesis of homogeneity of variance. Depending on the results of both tests, it was decided to perform statistical analyses with the parametric test of the analysis of variance or its non-parametric equivalent – Kruskal-Wallis rank ANOVA.

RESULTS

The mean number of eggs detected in samples of all types of soil by particular methods is presented in Table 1.

Table 1. Mean number of *Ascaris suum* eggs detected in samples by different methods (24 samples for each method), regardless of the type of soil examined

Method	Mean number of *Ascaris suum* eggs
Vasilkova	7.84
Dada	14.92
Quinn	2.67
PN-19000	4.25
PN-19005	24.67
PN-19006	30.17

The obtained results indicate that the most effective methods in the parasitological examination of various types of soil were the standardized methods PN-19006 and PN-19005. The lowest efficiency was obtained using Quinn and PN-19000 methods. The highest number of eggs were detected in SS, and the lowest in PS samples (Table 2).

The mean number of *A. suum* eggs detected in samples of each type of soil examined with particular methods, as well as the range of values and standard deviation, are presented in Table 3.

The presented data show that only the PN-19006, PN-19005 and Dada methods proved to be useful for examination of each type of soil. By each method, the largest number of eggs was detected from sandy soil samples, and the lowest from peat-based substrate (except for the PN-19006 method, which showed a similar number of eggs in horticultural mix soil and peat-based substrate). Most eggs were isolated from samples examined with the PN-19006 method. The mean number of nematode eggs found by this method in HS, SS and PS was 21.25, 46.50, and 23.00, respectively. Slightly lower results were obtained in the study using the PN-19005 method. The mean number of eggs detected by this method was 21.25, 36.00 and 16.75, respectively. On the other hand, the mean number of *A. suum* eggs detected in the samples tested with the Dada method was about 2–3 times lower than with the PN-19006 method and amounted to 15.75, 22.50 and 6.50, respectively, for HS, SS and PS.

Table 2. Mean number of *Ascaris suum* eggs detected in samples in different types of soil (48 samples for each type of soil), regardless of the method

Type of soil	Mean number of *Ascaris suum* eggs
HS	11.51
SS	22.59
PS	7.71

HS – horticultural mix soil; SS – sandy soil; PS – peat-based substrate

The method according to Vasilkova and Gefter, and the method according to Quinn, turned out to be much less effective in the detection of *A. suum* eggs from sandy and horticultural soil samples than the methods mentioned above. These methods proved to be completely ineffective in the case of testing peat-based substrate samples. The mean number of eggs isolated from sandy and horticultural mix soil samples using the Vasilkova method was 14.75 and 8.75, respectively, and in the case of the Quinn method – 4.50 and 3.50 eggs per sample, respectively.

The method according to PN-19000 proved to be the least effective in recovering parasite eggs from soil samples. When using this method, parasite eggs were isolated only from sandy soil samples. The average number of eggs isolated from this type of soil was 12.75, and the range from 10–18. No *Ascaris* eggs were detected in samples of peat substrate and horticultural soil using PN-19000 method.

Assuming the parameters of the most effective method – PN-19006 as 100%, the relative effectiveness of other methods in the study of particular types of soil was related to it. (Tab. 4).

The obtained results showed that the PN-19006 method was much more effective in the parasitological examination of all soil types used in the experiment. The PN-19005 method was 2–3 times less effective, which is mainly due to the twice smaller mass of the analytical sample used in this method. In the examination of various types of soil, the Dada method showed 2.83 – 7.41% of the effectiveness of the PN-19006 method (with 10 times less mass of the analytical sample). The PN-19000–4 method showed relatively low efficiency only in the case of SS, but it was more than 3 times lower than the effectiveness of the PN-19006 method.

The Lilliefors and Shapiro-Wilk tests confirmed the normal distribution of the trait – the number of detected eggs, but the Levene and Brown-Forsyth tests rejected the hypothesis of homogeneity of variance. For this reason, further analyzes were performed using Kruskal-Wallis non-parametric ANOVA of ranks. This test confirmed the statistical significance of differences in the number of eggs

Table 3. Mean number of *Ascaris suum* eggs detected in soil samples examined with different methods (8 samples for each variant)

Method	Mass of analitycal sample [g]	HS				SS				PS			
		No. of positive results	No. of detected eggs			No. of positive results	No. of detected eggs			No. of positive results	No. of detected eggs		
			Mean	Range	SD		Mean	Range	SD		Mean	Range	SD
Vasilkova	10	8	8.75	5–17	4.74	8	14.75	8–25	6.80	0	0.00	0	0.00
Dada	10	8	15.75	12–19	2.82	8	22.50	20–26	2.62	8	6.50	4–8	1.69
Quinn	50	8	3.50	3–5	0.76	8	4.50	1–9	3.16	0	0.00	0	0.00
PN-19000	100	0	0.00	0	0.00	8	12.75	10–18	3.41	0	0.00	0	0.00
PN-19005	50	8	21.25	15–30	5.44	8	36.00	28–41	4.87	8	16.75	11–27	6.50
PN-19006	100	8	21.25	15–28	4.65	8	46.50	38–52	5.01	8	23	17–28	4.71

HS – horticultural mix soil; SS – sandy soil; PS – peat-based substrate

Table 4. Relative efficiency of the compared methods (effectiveness of the PN-19006 method = 100%)

Method	Relative efficiency (%)		
	HS	*SS*	*PS*
PN-19006	100	100	100
Vasilkova	4.12	3.17	0
Dada	7.41	4.84	2.83
Quinn	8.24	4.84	0
PN-19000	0	27.42	0
PN-19005	50	38.71	36.41

HS – horticultural mix soil; SS – sandy soil; PS – peat-based substrate

Table 5. P-value for multiple (two-tailed) comparisons; for the number of eggs detected by different methods for horticultural mix soil (HS) Test Kruskal-Wallis: H (5, N= 48) =40.62696; p =0.0000

Method	PN-19000 R:4.5000	Vasilkova R:21.938	Quinn R:12.688	PN-19006 R:38.813	PN-19005 R:38.750	Dada R:30.313
PN-19000		0.191038	1.000000	***0.000014***	***0.000015***	***0.003397***
Vasilkova	0.191038		1.000000	0.238820	0.244729	1.000000
Quinn	1.000000	1.000000		***0.002848***	***0.002951***	0.177107
PN-19006	***0.000014***	0.238820	***0.002848***		1.000000	1.000000
PN-19005	***0.000015***	0.244729	***0.002951***	1.000000		1.000000
Dada	***0.003397***	1.000000	0.177107	1.000000	1.000000	

R -– average rank for the tested method

Table 6. P-value for multiple (two-tailed) comparisons; for the number of eggs detected by different methods for sandy soil (SS). Test Kruskal-Wallis: H (5, N= 48) =42.51265; p =0.0000

Method	PN-19000 R:16.188	Vasilkova R:17.688	Quinn R:4.9375	PN-19006 R:43.875	PN-19005 R:37.125	Dada R:27.188
PN-19000		1.000000	1.000000	***0.001146***	0.041700	1.000000
Vasilkova	1.000000		1.000000	***0.002749***	0.082349	1.000000
Quinn	1.000000	1.000000		***0.000000***	***0.000064***	***0.022200***
PN-19006	***0.001146***	***0.002749***	***0.000000***		1.000000	0.256934
PN-19005	***0.041700***	0.082349	***0.000064***	1.000000		1.000000
Dada	1.000000	1.000000	***0.022200***	0.256934	1.000000	

R -– average rank for the tested method

isolated by various methods: Kruskal-Wallis rank ANOVA: H (5, N = 144)=92.86501 p = 0.0000 and the number of eggs isolated from different types of soil: Kruskal-Wallis rank ANOVA; H (2, N = 144) = 32.42142; p = 0.0000. P-value for multiple (two-tailed) comparisons; for the number of eggs isolated by different methods for different types of soil are collected in Tables 5–7.

Table 7. P-value for multiple (two-tailed) comparisons; for the number of eggs detected by different methods for peat-based substrate (PS) Test Kruskal-Wallis: H (5, N= 48) =45.38414; p =0.0000

Method	PN-19000 R:12.500	Vasilkova R:12.500	Quinn R:12.500	PN-19006 R:43.000	PN-19005 R:38.000	Dada R:12.500
PN-19000		1.000000	1.000000	***0.000198***	***0.004044***	0.334065
Vasilkova	1.000000		1.000000	***0.000198***	***0.004044***	0.334065
Quinn	1.000000	1.000000		***0.000198***	***0.004044***	0.334065
PN-19006	***0.000198***	***0.000198***	***0.000198***		1.000000	0.574781
PN-19005	***0.004044***	***0.004044***	***0.004044***	1.000000		1.000000
Dada	0.334065	0.334065	0.334065	0.574781	1.000000	

R -– average rank for the tested method

As can be seen from the presented data, statistically significant differences were found primarily between the results obtained in the low efficiency methods (PN-19000–4, Vasilkova and Quinn) and the high efficiency methods (PN-19006 and PN-19005). The results obtained with the Dada method – a method of medium efficiency, did not differ statistically significantly from the results obtained with the use of most methods.

DISCUSSION

The study compared the effectiveness of own methods with other methods most often used in Poland for parasitological examination of soil. The experiments were carried out on soils/substrates differing primarily in the content of organic substances.

Ascaris eggs, which are the most common in cultivated soils in Poland, were used to spike soil samples [13]. The procedures used in similar studies assumed the addition of parasite eggs to laboratory samples (from which analytical samples are taken after thorough mixing) [6], or directly to analytical samples [14]. In own experimental methodology, the second procedure was chosen, using a fixed number of 100 eggs/sample, regardless of its mass (for various methods, the mass of analytical samples ranged from 10 – 100 g). Such a procedure made it possible to omit an error in the experiment resulting, e.g. from non-homogenous distribution of eggs in a laboratory sample, from which analytical samples would then be taken. The use of a constant and relatively high number of eggs in the analytical sample allowed for easy determination and reliable comparison of egg recovery with the use of individual methods.

It should be clearly emphasized here that the purpose of the study was not to determine the limit of detection

(LOD) of individual methods (as this would require a full validation process at many levels of sample spiking), but only a comparison of the usefulness (expressed by efficiency) of individual methods. In order to determine the actual effectiveness of individual methods, it was necessary to take into account the mass of analytical samples in the further analysis, assuming that the greater the mass of the analytical sample, the theoretically greater the effectiveness of the method. This relationship is directly proportional, especially at low contents of parasite eggs in the sample mass unit, which is found in natural conditions.

The methods for helminth eggs detection in soil selected in this study had a similar scheme of procedure. These were concentration methods based on the flotation process, preceded by the stage of loosening the sample structure ('rinsing') with detergent or alkaline solutions. In addition, in some methods (PN-19005 and PN-19006), after the flotation stage, an additional sedimentation stage is used to remove excess salt from the preparation before the sample incubation stage in order to determine the viability of eggs. These methods differ in the fluids used to loosen the structure of the sample, the type of flotation fluid, the ratio of the sample mass to the volume of the flotation fluid, or the support of the flotation process by centrifugation, the mass of the analytical sample, and the method of preparing the microscope slides. For example, in PN-19000 and Vasilkova methods, 5% NaOH is used to loosen the sample structure, and in the other methods – 0.0025–0.1% Tween 20. In the flotation stage, saturated salt solutions are used – Na_2NO_3 (Vasilkova, PN-19000, PN-19005 and PN-19006), NaCl (Quinn) and $ZnSO_4$ (Dada).

In all methods, the flotation process is supported by centrifugation, but the centrifugation force varies from 780 x g to 2,600 x g. The mass of the analytical sample is 10 g (Vasilkova and Dada), 50 g (Quinn and PN-19005) and 100 g (PN-19000 and PN-19006). In Dada and Quinn methods, the microscope slide is prepared using a coverslip – as in the traditional Fulleborn flotation method modified by Willis [15], while in the other methods a filter (paper or polycarbonate) is used, although the amount of filtered liquid (supernatant) is different.

The conducted experiments showed that the PN-19006 method was characterized by the highest percentage of recovered *A. suum* eggs (21–46%) from the analytical sample of various types of soil. This is the method used for parasitological examination of organic fertilizers. Slightly lower percentages of recovered eggs were obtained using the PN-19005 and Dada methods. However, analytical samples with a smaller mass are used in these methods (respectively, 50 g and 10 g), which in total makes their relative effectiveness lower than PN-19006 (from a few to several times).

Other methods showed very low efficiency, especially in the parasitological examination of samples with a high content of organic substances. Analyzing the low effectiveness of these methods, it should be noted that the NaOH solution used to loosen the sample structure, in the case of soils with a high content of organic substances may cause the opposite effect from the expected one. Namely, after adding the NaOH solution the structure of the HS and PS "swollen", and the solids of the samples increased in volume. Both methods, in which the stage of loosening the sample structure using NaOH solution (Vasilkova and PN-Z-19000) proved to be ineffective in the study of the peat-based substrate (PS). In the case of horticultural mix soil (HS), Vasilkova method proved to be low effective, and PN-19000 – completely ineffective. The low usefulness of the NaOH solution is also indicated by the study of Ruiz De Ybanez et al. [16]. According to the literature, the most useful solutions for rinsing samples are solutions of anionic detergents from the Tween group [14, 17]. These solutions were used in other methods, including the most effective method PN-19006.

In the case of the Quinn method, it seems that its low efficiency results from the low density of the used flotation fluid – a saturated NaCl solution (1.197 g/ml at room temperature). The authors of other flotation methods, including methods for parasitological soil testing, also indicated the low usefulness of the saturated NaCl solution [18]. Generally, the greater usefulness of solutions in higher density is emphasized [19, 20]. However, there are also studies showing that not only the density of the fluid is decisive for the efficiency of the flotation process [17], but also, for example, the viscosity of the fluid. In the case of some flotation solutions, problems may arise related to the rapid crystallization of salts on microscope slides prepared for examination. Such a phenomenon was observed by other authors, for example, using Na_2NO_3 solutions [17]. During the experiment described in this article, such a phenomenon was observed only when a $ZnSO_4$ saturated solution (Dada method) was used. This practically limits the possibility of using such a flotation solution. However, as indicated by the observations of Ajala et al. [19], replacing the observations on the coverslip with observations on the filter (with the addition of a few drops of glycerol on its surface) may completely eliminate this problem.

Own observations obtained during the development of a method for the parasitological examination of dehydrated sewage sludge showed that the centrifugation force supporting the flotation process and its duration are also of great importance when isolating eggs from the sample [21]. In this study, it was found that by increasing centrifugation force, the trend line of the function of the number of detected parasite eggs takes on the character of a logarithmic function, and satisfactory results are achieved only by centrifugation with a force of at least 2,000 x g for at least 10 min. For this reason, it appears that the centrifugation force used in the PN-19000 (780 x g) and Dada (1500 x g) methods is too low, which may negatively affect the performance of both methods. Similarly, the centrifugation time in the PN-19000 method (3 min) and Vasilkova's (2 min) is probably too short.

A very important factor that may affect the effectiveness of flotation methods is the ratio of the mass of the analytical sample to the volume of the fluid used for flotation. According to the cited study [21], the best detection of parasite eggs in the flotation process was obtained when the ratio of the sample mass and the volume of the flotation fluid was 1:32, or more. In the case of using centrifugation in the flotation process, satisfactory results were obtained with a ratio 1:4–1:8. Therefore, it seems that, for example, in the PN-19000 method, the inappropriate ratio of the sample mass to the volume of the flotation fluid may be the main reason for its low efficiency.

CONCLUSIONS

The study shows that the own method (PN-19006) is the most useful for soil examination among all compared methods. Its egg recovery rate is very high, ranging from 46.5% in sandy soil up to 21.25% for soil with a high content of organic matter. Other authors, validating their methods, usually obtained lower rates of recovery of geohelminth eggs. For example, Santarem et al. recovered 4.25–10.75% of *T. canis* eggs from sandy soil [17], Oge et al. up to 15% [14], and Ajala et al. [19] recovered 13.88–25.04% *A. lumbricoides* eggs. A slightly higher percentage of recovered *Ascaris* eggs was obtained by Cranston et al. – 10.1–36.5% [12]. At the same time, assuming a large mass of the analytical sample used in the PN-19006 method, it can be assumed that this method is useful for the examination of soil samples, or at least can be used as a basis for developing specific methods for parasitological examination of soil. It should be noted, however, that the degree of recovery of *A. suum* eggs largely depends on the type of soil which, in the opinion of the authors of this study, makes it impossible to use one method as universal for all types of soil, and certainly prevents full validation of the method assuming its universality. Therefore, it seems advisable to develop a system of parasitological methods for examination of different types of soils, and linking them with a system of conversion factors enabling comparison of the obtained results.

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