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Comparison of *Cryptosporidium* oocyst recovery methods for their applicability for monitoring of consumer-ready fresh shellfish

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ABSTRACT

Growing demand for fresh, unprocessed food favours the emergence of *Cryptosporidium* infections in humans. Mainly it is food of plant origin or unpasteurized milk which have been involved in food-borne outbreaks of cryptosporidiosis. So far consumption of shellfish contaminated with *Cryptosporidium* were not associated with human infections although such as possibility exists. In this study an attempt was undertaken to evaluate the analytical performance of three commonly used methods for recovery of *Cryptosporidium* oocysts from shellfish: i) pepsin digestion of shellfish in conjunction with immunomagnetic separation (IMS) of oocysts (method A), ii) pepsin-HCl treatment of shellfish homogenate without IMS (method B), and iii) a strainer method with direct oocyst extraction and separation from shellfish tissue using IMS (method C). Each method's performance was assessed according to the ISO standard requirements by testing shellfish homogenates seeded with different numbers of *C. parvum* oocysts. Two groups of parameters were compared, encompassing precision (coefficient of variation (CV)) and accuracy of measurements. These were described by linear regression models allowing calculation of the methods' limits of detection (LOD) and quantification (LOQ). In addition, oocyst recovery efficiencies from shellfish were calculated for each method. All three compared methods allowed for at least 66% recovery of *Cryptosporidium* oocysts from the tested samples. The best recovery (83.3–100%) in the whole range of tested suspensions was obtained for method C. The accuracy of method B was better (linearity of $r^2 = 0.9996$ in the full measurement range) than that of method A ($r^2 = 0.968$). Method C showed the best accuracy ($r^2 = 1$) and precision (CV 0.2–14.1). Compared to other methods it was also characterised by the best LOD and LOQ, attaining ≅4 and ≅12 oocysts per 3 g of tested shellfish sample respectively. Despite a lack of the ability of method A to give the proportional results in oocysts recovery (non-linearity of the method) compared to the reference values, it achieved the highest LOD and LOQ values among the tested methods. As demonstrated here, the most efficient method for extraction of *Cryptosporidium* oocysts from shellfish tissues was method C employing sample homogenisation and separation of oocysts from tissue debris using IMS. Used alone this method does not in fact allow for identification of *Cryptosporidium* species but delivers quantitative results concerning the level of food contamination by parasites.

1. Introduction

Growing demand for fresh, unprocessed food favours the emergence of *Cryptosporidium* infections in humans. Leafy green vegetables (Insulander et al., 2008), fresh fruit juices (Blackburn et al., 2006; Millard et al., 1994) and unpasteurized milk (Gelletlie et al., 1997; Harper et al., 2002; Rosenthal et al., 2015) are the major food types which consumption has led to outbreaks of cryptosporidiosis. Although consumption of *Cryptosporidium*-contaminated shellfish has not been shown to produce outbreaks of human cryptosporidiosis (Ahmed and Karanis, 2018a), the frequent occurrence and long survival of *Cryptosporidium* in this food type suggests the need for further surveillance studies. Attribution of human outbreaks to *Cryptosporidium* is not altogether unlikely because there is still little knowledge about the role of shellfish in food-borne transmission of *Cryptosporidium* to humans. Mussels are filter-feeding organisms, therefore besides nutritional ingredients they can absorb and accumulate different biological contaminants from the surrounding water (Leal et al., 2013; Souza et al., 2013). Besides viruses and bacteria infectious for humans, shellfish also harbour different species of protozoan parasites such as *Giardia* (Coupe et al., 2018; Ghozzi et al., 2017; Giangaspero et al., 2014; Ladeiro et al., 2014; Marangi et al., 2015), *Toxoplasma* (Aksoy et al., 2014; Coupe

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et al., 2018; Ghozzi et al., 2017; Ladeiro et al., 2014; Marangi et al., 2015; Putignani et al., 2011; Staggs et al., 2015), *Cyclospora* (Aksoy et al., 2014; Ghozzi et al., 2017), *Encephalitozoon* (Graczyk et al., 2004; Lucy et al., 2008) and *Cryptosporidium* (Giangaspero et al., 2014; Leoni et al., 2007; Marangi et al., 2015; Miller et al., 2005b; Oliveira et al., 2016; Schets et al., 2013). The prevalence and level of shellfish contamination with *Cryptosporidium* oocysts depend on the microbiological quality of shellfish growing waters and the type of tested mollusc species (Giangaspero et al., 2014; Tei et al., 2016). Their occurrence in shellfish grown in Europe ranges from 0.36% to 56.7% (Giangaspero et al., 2014; Molini et al., 2007). Despite the frequent detection of *Cryptosporidium* oocysts in shellfish at the time of harvesting from officially classified waters, their occurrence in molluscs is generally low when the products are tested later at the consumer-ready stage. This may indicate an efficient depuration procedure before sale or a low efficiency of extraction and concentration methods employed for testing of *Cryptosporidium* contaminants in food.

Currently the methodologies used for *Cryptosporidium* detection in shellfish consist of two main steps: i) concentration of oocysts from shellfish (whole animals, digestive glands or hemolymph), and ii) detection of parasites using microscopy (Gómez-Couso et al., 2006; Robertson and Gjerde, 2008; Willis et al., 2014) or their genomic DNA by molecular methods (Giangaspero et al., 2014; Gómez-Couso et al., 2006; Srisuphanunt et al., 2009; Staggs et al., 2015). Before *Cryptosporidium* oocysts can be detected, they have to be liberated and removed from the remaining food solids and concentrated. It can be achieved by several treatments involving initial sample homogenisation and pepsin digestion of shellfish tissues or by using a bead-based immunomagnetic separation (IMS) tool. Although the concentration procedures seem to be universal, allowing the processing of various shellfish, differences in oocyst recoveries are observed. The concentration methods compared in this study have previously proved their applicability in testing of shellfish samples for the presence of *Cryptosporidium* oocysts, although their diagnostic performance was not fully evaluated. Despite great advances and achievements have been made in the development of the methods for detection of *Cryptosporidium* in food (Ahmed and Karanis, 2018a), water (Efstratiou et al., 2017) and clinical material (Ahmed and Karanis, 2018b) there are still not sufficient data available on validation of methods or their harmonisation among different laboratories. The use of standardized protocols in parasite detection will better define the real epidemiological situation of cryptosporidiosis worldwide (Ahmed and Karanis, 2018b). Likewise to conduct epidemiological studies or to assess the risk of infection related to consumption of contaminated shellfish efficient *Cryptosporidium* oocyst recovery methods are needed. Thus, the aim of this study was an evaluation and statistical assessment and comparison of the analytical methods' performance to ensure that their validation parameters fit the criteria for an efficient method for parasite recovery from shellfish.

2. Material and methods

2.1. Shellfish and Cryptosporidium oocysts

A batch of blue mussels (*Mytilus edulis*) originating from the Netherlands were purchased from a domestic seafood wholesaler. Shells of individual mussels were opened using a sterile scalpel after cutting of the adductor muscle and then flesh was removed. A pool (1 kg) of whole shellfish homogenate was prepared on ice using an Omni TH 220-PCRH mechanical tissue homogenizer (Omni International, USA). Subsequently, shellfish homogenate was divided into 3.0 (± 0.05) g testing portions. Stock suspension of *Cryptosporidium parvum* (*C. parvum*) oocysts of calf origin at concentration of 1×10^7/8 ml (Iowa strain, Waterborne, Inc., USA) were used for seeding experiments of shellfish homogenate samples during the method comparison trial.

2.2. Seeding of shellfish homogenate samples with Cryptosporidium oocysts

Before determination of the efficiency of each method's recovery of *Cryptosporidium* oocysts from shellfish, a 3 g portion of blue mussels taken from the homogenate pool was initially tested for natural *Cryptosporidium* contamination. The method described by Robertson and Gjerde (2008) was initially employed for parasite recovery, with subsequent detection of oocysts using microscopy. Subsequently, shellfish homogenate portions were contaminated with 1 ml suspensions of *C. parvum* oocysts (Iowa strain) containing 10 (± 1), 50 (± 2), 100 (± 2), 500 (± 6) and 1000 (± 10) oocysts respectively. Seeding suspensions were prepared according to ISO 18744 method (Anonymous, 2016a). The homogeneity of oocyst suspensions (the real number of oocysts at each level of contamination) was verified by testing three replicates representing each level of contamination using epifluorescence microscopy. Briefly, for each control suspension containing a defined number of oocysts the coefficient of variation (CV) was calculated. It was defined as the ratio of the standard deviation calculated from the number of *Cryptosporidium* oocysts found in suspension to the average number of oocysts present at the given level of contamination. CV values ≤ 16% were considered satisfactory and indicated the homogeneity of oocyst suspensions used for assessment of the methods' recovery efficiencies.

2.3. Methods comparison

The recovery efficiencies of *Cryptosporidium* oocysts for the tested methods were assessed based on the number of detected oocysts in 3 g portions of shellfish homogenate seeded with 10, 50, 100, 500 and 1000 *C. parvum* oocysts. For each level of contamination, the test was carried out in 6 replicates. *Cryptosporidium* oocysts were extracted and separated from shellfish homogenate portions using the following methods: i) the method of Robertson and Gjerde (2008) utilising pepsin digestion of shellfish in conjunction with immunomagnetic separation (IMS) of oocysts (*method A*), ii) the method employing of pepsin-HCl treatment without the application of IMS described by Willis et al. (2014) (*method B*), and iii) a strainer method with direct oocyst extraction and separation from shellfish tissue debris using IMS (*method C*; Miller et al., 2005a). The percentage of recovered *Cryptosporidium* oocysts was determined based on the number of FITC-*C*-mAb stained oocysts recovered from each sample in comparison to the number of oocysts present in seeding suspensions.

2.3.1. Method (A)

13 ml of a pepsin digestion solution (1% pepsin, 1:10,000 NF) was added to 3 g of shellfish homogenate and the mixture was heated at 37 °C for 1 h and stirred every 20 min. The suspension was centrifuged at 13,000 ×*g* for 1 min, the supernatant was discarded, and the remaining pellet was rinsed twice and subsequently resuspended in 1 ml of distilled water (pH 7.0). The suspension was transferred to a Leighton tube and the sample volume was increased to 10 ml using distilled water. The IMS on shellfish homogenate samples was performed using the Crypto-Scan IMS test kit (TCS Water Sciences, UK) according to the manufacturer's instructions. The obtained eluate (50 µl) was transferred onto an immunofluorescence microscopy slide (Medlab, Poland), and air-dried at room temperature.

2.3.2. Method (B)

Shellfish homogenate (3 g) was incubated at 35 °C for 1.5 h in 25 ml of digestion solution (1% pepsin, 1:10,000 NF) with continuous mixing at 150 rpm in an air shaker (Lab-Line, Thermolyne, USA). Then the suspension was centrifuged at 900 ×*g* for 5 min. The supernatant was discarded and the pellet was rinsed respectively with distilled water (pH 7.0) and PBS (pH 7.4). The pellet was resuspended in 50 µl PBS and transferred onto the microscope slide.

Table 1
Recovery efficiencies of the methods.

Level of contamination (number of oocysts)	ANOVA summary	Control suspensions	Method A			Method B			Method C		
		Number of oocysts (SD)	Number of oocysts (SD)	Recovery (%)	p*	Number of oocysts (SD)	Recovery (%)	p*	Number of oocysts (SD)	Recovery (%)	p*
10	F = 5.2, p = 0.010	9 (± 1)^{A}	7 (± 2)^{B}	74.1	0.024	6 (± 1)^{B}	66.7	0.019	8 (± 15)^{A}	83.3	1.000
50	F = 2.3, p = 0.114**	50 (± 1)	42 (± 2)	83.5	–	37 (± 15)	72.9	–	46 (± 1)	92	–
100	F = 52.4, p < 0.0001	97 (± 2)^{A}	86 (± 3)^{B}	88.6	< 0.0001	86 (± 2)^{B}	88.7	< 0.0001	97 (± 1)^{A}	100	0.957
500	F = 60.0, p < 0.0001	493 (± 2)^{A}	338 (± 9)	68.5	< 0.0001	466 (± 38)^{A}	90.4	0.206	493 (± 2)^{A}	100	1.000
1000	F = 17.8, p < 0.0001	889 (± 4)^{A}	839 (± 13)^{B}	94.3	0.0003	835 (± 27)^{B}	93.9	0.0002	889 (± 2)^{A}	100	0.975

A, B – Letters indicate statistical consistency (Duncan *post hoc* test, p > 0.05) between oocyst recovery obtained for compared methods and oocyst content in control suspensions.

* Probability values (Duncan *post hoc* test, p ≤ 0.05) obtained for each assessed method indicate statistically significant differences in the oocyst recovery compared to oocyst content in control suspensions at a given level of contamination. Results equal or close to 1 indicate their concordance with level of contamination for control suspensions.

** There are no statistically significant differences (ANOVA) between the results of oocyst recovery obtained for compared methods and oocyst content in control suspensions.

2.3.3. Method (C)

Shellfish homogenate (3 g) was sieved through a 100 µm cell strainer and the remaining semi-liquid homogenate was centrifuged at 1000 ×*g* for 15 min. Supernatant was carefully removed and the resulting sediment was subjected to the IMS procedure and microscopic examination as for method A.

2.4. Detection of Cryptosporidium oocysts

A direct immunofluorescence antibody (IFA) assay was applied to detect and determine the number of *Cryptosporidium* oocysts in shellfish sediments and control suspensions. Slides containing air-dried, methanol-fixed sample extracts, were stained with a FITC-conjugated monoclonal antibody (Crypt-a-Glo FITC-*C*-mAb, Waterborne Inc., USA) and with the fluorogenic DNA intercalator 4′,6 diamidino-2-phenylindole (DAPI) (Grimason et al., 1994; Smith et al., 2002). Samples were mounted in 60:40 glycerol (PBS containing 2% (w/v) of the antifadant 1,4-diazabicyclo[2,2,2]octane). Each microscope slide was covered by a glass coverslip, which was sealed onto the slide using clear nail varnish and viewed within 30 min of preparation. Microscopy was performed using an Olympus BH2 fluorescence microscope (×40 and ×100 lenses, ×12.5 eyepiece), equipped with Nomarski differential interference contrast (DIC) optics. A blue filter (excitation 480 nm, emission 520 nm) was used for the detection of FITC-conjugated mAb-labelled oocysts and a UV filter block for DAPI (excitation 350 nm, emission 450 nm). At the same time, the control microscope slides were prepared containing i) a sample eluate from shellfish homogenate seeded with a known number of oocysts and ii) a positive control of the staining procedure. The laboratory operates within a quality management system and is ISO 17025:2005 accredited.

2.5. Assessment of the method performance

Each method's performance was assessed based on the comparison of two groups of method parameters, *i.e.* precision and accuracy (Anonymous, 2016b, c; OIE, 2017). The accuracy was defined by the degree of match between the number of recovered oocysts and the number of oocysts present in the control suspension at a given level of contamination. The precision was established as the dispersion of results obtained for the tested methods at each contamination level, including an assessment of measurement coefficients of variation (CV values). CVs ≤ 16% indicated high precision of the method, CVs ranging from 16% to 32% signified lower precision, while CVs above 32% showed unacceptable precision. A method's accuracy was assessed based on the evaluation of the appropriateness of the regression model (r^2, a, b) which defines the ability of the method to obtain proportional results when an increased number of oocysts in control suspensions is tested. The accuracy and bias of the method are defined as the difference between the assumed level of contamination and the result obtained for the tested method. The developed linear regression models were used to calculate the detection (LOD) and quantification (LOQ) limits of the methods (Shrivastava and Gupta, 2011). The LOD and LOQ parameters were calculated using the equations $LOD = 3S_a/b$ and $LOQ = 10S_a/b$, where S_a is the standard deviation of the response and b is the slope value of the regression curve.

The differences in oocyst recoveries for the compared methods at a given level of contamination were analyzed using one-way analysis of variance (ANOVA) with F statistics used as measure of differences observed. Duncan's *post hoc* test was used to assess mutual differences between values of oocyst recoveries obtained for each method and control suspensions (reference material) containing *Cryptosporidium*. The statistical significance for all tests was $p = \alpha = p \leq 0.05$. All calculations were performed with Statistica v. 10 (Statsoft, USA).

3. Results

3.1. Method recovery efficiencies

All compared methods allowed for at least 66% recovery of *Cryptosporidium* oocysts from the tested samples. The methods involving pepsin treatment of shellfish tissues (methods A and B) gave similar recovery efficiency only at contamination levels of 10 ($p < 0.024$), 100 ($p < 0.0001$), and 1000 ($p < 0.0003$) oocysts (Table 1). There were no differences observed between methods when suspensions containing 50 oocysts were analyzed ($p = 0.114$). At the contamination level of 500, the oocyst recovery obtained for method A differed significantly (68.5% recovery, $p < 0.0001$) from those for method B (90.4% recovery, $p = 0.206$), method C (100% recovery, $p = 1.0$) and control suspensions containing defined number of oocysts.

As compared to other methods, the most efficient oocyst recovery (the highest number of oocysts in the whole range of tested contamination levels) was observed for method C with direct separation of oocysts from shellfish tissues using IMS (Fig. 1). For samples spiked with 10 and 50 oocysts the recoveries ranged from 83 to 92%, whereas 100% of oocysts were recovered from samples containing 100, 500 and 1000 oocysts (Table 1). The Duncan *post hoc* test showed that obtained *Cryptosporidium* recoveries for method C were consistent with the number of oocysts present in control suspensions at each level of contamination ($p = 0.957–1.0$). Only method B gave a similar recovery to method C when shellfish samples contained 50 and 500 oocysts

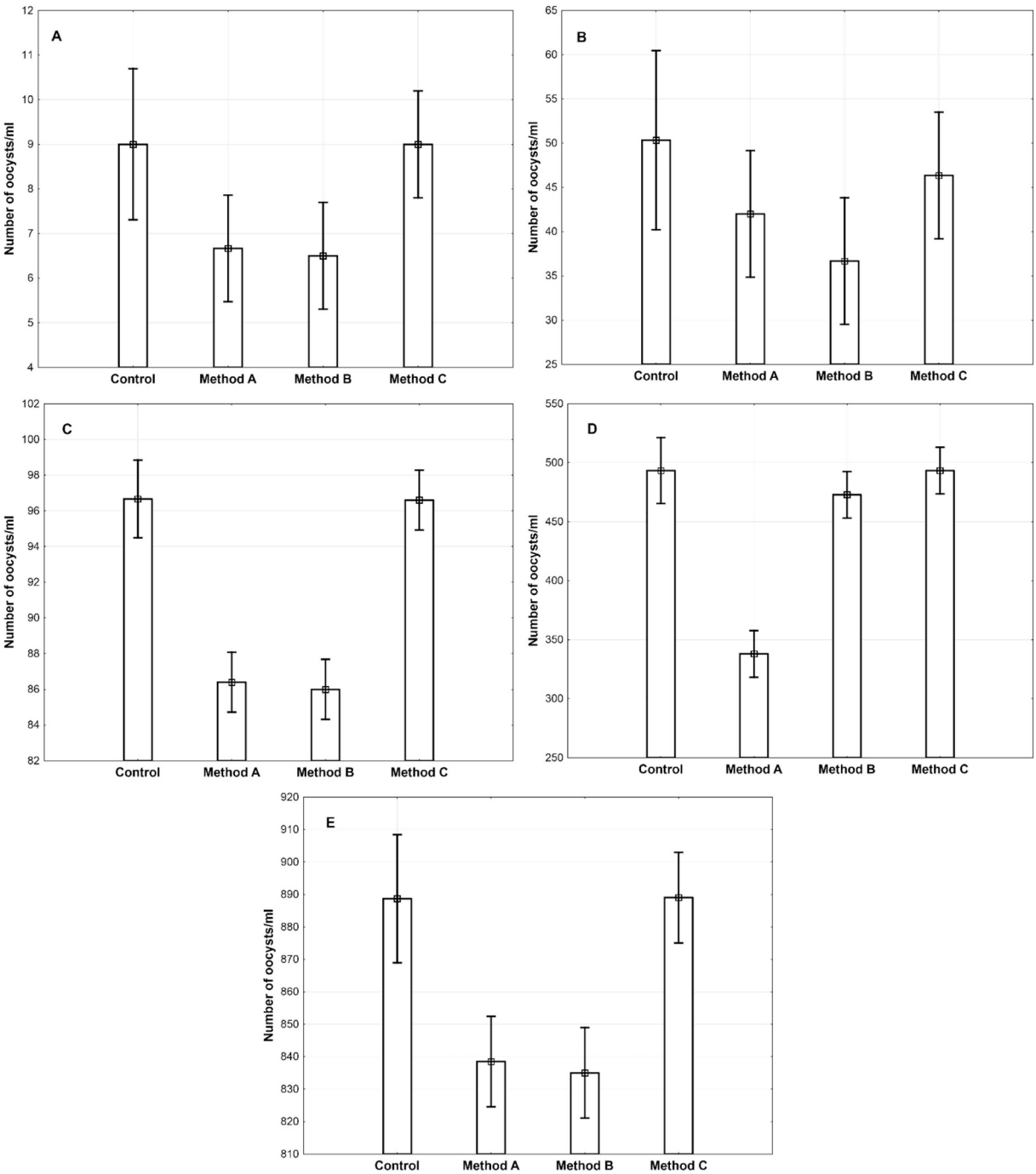

Fig. 1. Graphical representation of the methods' recovery efficiencies at the following levels of contamination: A – 10 oocysts, B – 50 oocysts, C – 100 oocysts, D – 500 oocysts and E – 1000 oocysts.

Table 2
Results on method precision.

Method	Contamination levels[a]/CV(%)				
	10	50	100	500	1000
Method A	15.5	5.6	2.7	2.7	1.6
Method B	28.8	41.1	2.0	8.7	3.2
Method C	14.1	2.6	1.2	0.4	0.2
Control suspensions	11.1	2.3	1.6	0.4	0.4
Assessed number of oocysts	9	50.3	96.7	493.3	889

CV – The method's precision expressed as a percentage value.
[a] 10–1000 represent numbers of oocysts at each level of contamination.

($p = 0.206$).

3.2. Precision and accuracy of the methods

CVs obtained for control suspensions of *Cryptosporidium* oocysts did not exceed 16%, which confirmed their homogeneity at each level of contamination (Table 2). The best precision with the lowest CV values for a given level of contamination was achieved for method C. Method A was also characterised by a good CV of ≤15.5%. In the case of method B, satisfactory precision (CV ≤ 8.7%) for tested suspensions containing at least 100 oocysts was obtained. The best linearity in the whole range of tested suspensions was obtained for method C, $y = -1.39 + 1.002x$ with $p = 3.4 \times 10^{-8}$. The accuracy of method B ($r^2 = 0.9996$) was better than that of method A ($r^2 = 0.968$), although method A was characterised by better precision. The regression equations and their parameters are presented in Table 3.

By the use of linear regression the following method LODs were determined: 125 oocysts for method A, ≅14 oocysts for method B, and ≅4 oocysts for method C (Table 3). Among compared methods method C had the best LOQ (≅12 oocysts). In the case of methods A and B the LOQs allowed detection of the presence of at least ≅415 and ≅46 oocysts per 3 g of tested shellfish sample respectively.

4. Discussion

Prior to their adoption for routine microbiological food testing, analytical methods require evaluation of their diagnostic performance to confirm their suitability. Currently there are no available performance criteria or evaluation requirements for gauging candidate methods for the detection of parasites in food. However, these criteria do exist for methods detecting bacterial contaminants in food (Anonymous, 2016b, c). In this study an attempt was undertaken to evaluate the analytical performance of methods used for *Cryptosporidium* recovery from shellfish homogenates seeded with *C. parvum* oocysts according to the requirements described in the microbiological ISO standards. In this light, the methods' performances were assessed based on the comparison of their precision and accuracy with calculated LOD and LOQ limits. In addition, the homogeneity (CV values) of control suspensions containing *Cryptosporidium* oocysts used in seeding experiments was assessed. The results indicated that all suspensions were homogenous including those with the lowest number of oocysts (CV < 16%).

The best recovery efficiencies (83.3–100%) in the whole range of seeded shellfish homogenates were obtained for method C (the IMS-DFA method). As seen in a previous study, this method was also characterised by a good oocyst recovery rate when digestive glands of clams were processed instead of whole shellfish tissue homogenates (Miller et al., 2005a). Unlike our observations in which method A achieved 94.3% recovery, in the studies of Robertson and Gjerde (2008) when used for processing horse mussels the method was less efficient (68–79%). Compared to the results obtained by Willis et al. (2014), slightly better oocyst recovery (88.7–93.9%) was observed for method B but only for samples spiked with at least 100 oocysts. The observed differences in the efficiencies of parasite recovery could result from processing of different shellfish species, as methods B and C were initially developed for oyster testing. This relation was also shown in experiments aiming at determination of the recovery efficiency of *Cryptosporidium* oocysts from seeded bivalve molluscs (Schets et al., 2013). Experiments revealed that *Cryptosporidium* was significantly more efficiently recovered from mussels than from oysters. The number of recovered oocysts from shellfish can be influenced by more than the type of processed shellfish, by such as shellfish consistency, *e.g.* firmness (MacRae et al., 2005), sample size (Schets et al., 2013), sample type *i.e.* whole animal or dissected organs (Adell et al., 2014; Downey and Graczyk, 2007; Giangaspero et al., 2005; Gómez-Couso et al., 2003; MacRae et al., 2005; Srisuphanunt et al., 2009), the method of its initial processing (Downey and Graczyk, 2007; Schets et al., 2013), and changes of antigenic composition of the oocyst wall (Gómez-Couso et al., 2006). Likewise, the IMS effectiveness in the capture and release of oocysts differs and is dependent on various physicochemical properties of beads and food matrices (Ahmed and Karanis, 2018a). Nevertheless, the utility of IMS-DFA (method C) and method A in *Cryptosporidium* detection was manifested in clams originating from contaminated freshwater ecosystems in California (Miller et al., 2005a, b) and in shellfish collected from Norwegian coastal waters (Robertson and Gjerde, 2008). It has also been shown that in methods dedicated to *Cryptosporidium* detection in food and environmental matrices, IMS increased the detection limit of the method (Di Pinto and Tantillo, 2002; Downey and Graczyk, 2007; Gao et al., 2013; Lowery et al., 2001; Sunnotel et al., 2006; Ware et al., 2003). Although the incorporation of IMS in procedures aiming at *Cryptosporidium* detection in foods is highly justified, it is not essential, as initial sample processing seems to be more crucial for obtaining good recovery efficiency. Besides this, IMS is expensive and time-consuming extending the whole procedure. In this light, IMS on pepsin-treated shellfish homogenate (method A) compared to a procedure involving only pepsin-HCl digestion (method B) did not result in better precision, accuracy or LOQ. Successful detection of *Cryptosporidium* in digestive glands and gills of consumer-ready shellfish which were subjected to simple homogenisation without subsequent IMS processing has been shown by other authors (Giangaspero et al., 2014; Gómez-Couso et al., 2006; Srisuphanunt et al., 2009).

Analysing all method parameters such as precision, accuracy and

Table 3
Parameters of the linear regression model with estimated limits of detection (LOD) and quantification (LOQ) of the methods.

Method	Parameters					
	Linear regression equation y = a + bx	Coefficient of determination r^2	Statistics F	p-Value	LOD[a]	LOQ[a]
Method A	$y = -16.74 + 0.907x$	0.968	121.5	0.0016	124.4	414.5
Method B	$y = -4.84 + 0.950x$	0.9996	10,250	0.000002	13.2	45.3
Method C	$y = -1.39 + 1.002x$	1	1.6×10^5	3.4×10^{-8}	3.4	11.4

a – The intercept (where the line crosses the y-axis).
b – The slope of the regression curve.
[a] Estimated values using linear regression model.

LOQ, method C appeared to be superior to the other methods in detection and quantification of *Cryptosporidium* oocysts. For this method, the number of recovered oocysts was close to the actual number of oocysts present in suspensions ($r^2 = 1$). The accuracy and linearity of method B ($r^2 = 0.9996$), was better than for method A ($r^2 = 0.968$), although its precision was lower (CV = 28.8–41.1). Method A was characterised by nonlinearity regardless of the level of contamination of the tested suspension. Despite a lower LOQ (45 oocysts) estimated by the regression model, method B showed good precision (CV = 2.0–8.7) only for samples containing at least 100 *Cryptosporidium* oocysts. Shellfish depuration or relaying are commonly used methods for removal of bacterial and viral contaminants after mollusc harvesting (Love et al., 2010; McLeod et al., 2017). These processes can also significantly reduce the level of shellfish contamination with *Cryptosporidium* oocysts (Izumi et al., 2006; Willis et al., 2014). Therefore testing of shellfish with a low expected level of contamination (*e.g.* consumer-ready shellfish) requires an efficient and sensitive method. In fact, all the compared methods were able to detect 10 oocysts in shellfish homogenates, which indicates that their LODs were below this level. In comparison to other methods, method A was characterised by higher LOD and LOQ due to nonlinearity of the method resulting from lower oocyst recovery for samples contaminated with 500 oocysts. In this light, LOD is a critical factor for accurate detection of a low number of oocysts present in shellfish.

Infectivity or viability of parasites isolated from shellfish using the compared methods was not determined in this study. However, a maximum 23% decrease in viability of *C. parvum* oocysts (for method A) has previously been observed by Robertson and Gjerde (2008). Although the vital dye assays demonstrated that viability of parasites was affected, this finding was not unequivocally confirmed by fluorescent *in-situ* hybridization (FISH). Interpretation of results on the methods recovery should be referred to mussels as representative shellfish species chosen for studies. Mussels were used because they are commonly farmed and eaten shellfish species in Europe (FAO, 2018) including Poland (CSO, 2015; Kukułowicz and Brzeski, 2013). It is highly likely that differences in oocyst recoveries between compared methods will appear if other shellfish species are used as the food matrix. It has to be noted that method C was originally employed for testing clams, however in this study its good performance was also confirmed for mussels. The homogeneity of control suspensions used for the assessment of method parameters is important if the most reliable results are to be expected. Therefore it is advisable that the CV values obtained at each contamination level for the compared methods be statistically correlated to the normal distribution. Despite the small number of replicates tested, this requirement was still fulfilled and data on homogeneity of variances were obtained which were sufficient to permit use of one-way ANOVA. A crucial criterion of quantitative performance in comparison of the methods is the recovery efficiency. The seeding procedure only requires the use of a low number of oocysts, *i.e.* 10 to 1000, to obtain accurate measurement of recovery efficiency (Efstratiou et al., 2017). In this light, the studies described are in line with the seeding recommendation and the results obtained are statistically sound. This study assessed the performance of different *Cryptosporidium* recovery methods from shellfish. As demonstrated here, differences in methods' performances were observed despite processing the same type of samples. Even taking these into consideration all methods performed well and showed their diagnostic usefulness (with some limitations) in future monitoring or epidemiological studies on *Cryptosporidium* occurrence in shellfish. Nevertheless, the most efficient was the method employing initial sample homogenisation and sieving followed by oocyst centrifugation and separation using IMS. Used alone this method does not in fact allow for identification of *Cryptosporidium* species but delivers quantitative results concerning level of food contamination by parasites. Assuming that molecular methods such as PCR and DNA sequencing need to be employed for identification of the parasite at the species level.

Declarations of interest

Declarations of interest: none.

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