J Food Sci Technol (November 2019) 56(11):5000–5008
https://doi.org/10.1007/s13197-019-03972-7

ORIGINAL ARTICLE

Efficiency of the Q3 lab-on-chip Real Time-PCR platform for detecting protozoan pathogens in bivalve mollusks

Annunziata Giangaspero[1] · Marianna Marangi[1] · Maria Stefania Latrofa[2] · Giada Annoscia[2] · Lorenza Putignani[3] · Gioia Capelli[4] · Lucia Bonassisa[5] · Giovanni Normanno[1] · Domenico Otranto[2] · Marco Cereda[6] · Francesco Ferrara[6]

Revised: 13 June 2019 / Accepted: 19 July 2019 / Published online: 1 August 2019

Abstract The zoonotic protozoan parasites *Toxoplasma gondii, Cryptosporidium parvum* and *Giardia duodenalis* have been recorded worldwide in economically important edible shellfish, and are thus likely to represent a significant public health risk. Therefore, an innovative, user-friendly diagnostic tool is required in order to improve food safety control. The Q3 system is a miniaturized platform whose efficiency and applicability were investigated and compared with results obtained using standard Real-Time PCR. Tanks of saltwater containing acclimated *Mytilus galloprovincialis*, *Ruditapes philippinarum* and *Ostrea edulis* specimens were spiked with purified *Cryptosporidium, Giardia* and *Toxoplasma* cysts/oocysts at different concentrations (i.e., 10^3, 10^4 and 10^5). We then collected 30 specimens for each shellfish species from each group at 24 h and 72 h post-contamination. After DNA extraction, we tested all samples by Real-Time-PCR and Q3, and evaluated the sensitivity, specificity, predictive values, repeatability and concordance between the two systems. Concordance between Real-Time-PCR and Q3 was very good ($p < 0.01$), especially for *Toxoplasma* in *M. galloprovincialis* at both 24 h and 72 h after contamination, and in *O. edulis* at 72 h. The ability of Q3 to detect all the investigated pathogens was similar to that of Real-Time-PCR, and Q3 was efficient in detecting *Toxoplasma* in both *M. galloprovincialis* and *O. edulis*. This is the first study concerning the use of lab-on-chip technology in a food matrix, and in edible marine mollusks in particular.

Keywords Protozoans · Shellfish · Real-Time PCR · Lab-on-chip efficiency · Food safety

Introduction

Bivalve mollusks are an important food resource worldwide (FAO-WHO 2014a), and the annual world production of farmed food mollusks is 16.4 million tonnes, i.e. 21% of the entire aquaculture sector (FAO 2015). The main species farmed in the EU are mussels (mostly *Mytilus galloprovincialis*), at 328,000 tonnes (26% of total EU production), followed by oysters (*Ostrea edulis*) at 138,000 tonnes (11% of total EU production), and clams (*Ruditapes* spp.), at over 60,000 tonnes (Eurostat 2015).

In addition to the pathogens and contaminants defined by the current EU legislation regarding the sanitary quality and control of mollusks (Reg. EC 853/2004; Reg. EC 854/2004; Reg. EC 882/2004; Reg EC 1881/2006; Reg. EC 2285/2015), the protozoans *Giardia*, *Cryptosporidium* and *Toxoplasma* are important agents whose environmental

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13197-019-03972-7) contains supplementary material, which is available to authorized users.

✉ Annunziata Giangaspero
annunziata.giangaspero@unifg.it

[1] Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, Via Napoli 25, 71121 Foggia, Italy

[2] Dipartimento di Medicina Veterinaria, Università di Bari, Via Casamassima Km 3, Valenzano, 70010 Bari, Italy

[3] Unità di Parassitologia e Unità di Ricerca di Microbioma umano, Bambino Gesù, Ospedale Pediatrico e Istituto di Ricerca, Viale Di San Paolo, 15, 00146 Rome, Italy

[4] Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, 10, 35020 Legnaro, Padua, Italy

[5] BonassisaLab, Z.I. Km. 684.300 Zona Industriale Asi, Incoronata, 71122 Foggia, Italy

[6] STMicroelectronics Srl, Advanced System Technology, Via Camillo Olivetti, 2, 20864 Agrate Brianza, Italy

dissemination is closely associated with fecal matter of human and/or animal origin (Thompson et al. 2005; Dubey and Jones 2008; Robert-Gangneux and Dardé 2012; Mmbaga and Houpt 2017). When these protozoans are discharged into rivers via farming and urban wastewater or run-off water, they subsequently contaminate coastal waters and are filtered and concentrated by mollusks. It has been shown that *Giardia, Cryptosporidium,* and *Toxoplasma* oo/cysts are present in farmed mollusks (including *M. galloprovincialis, R. philippinarum,* and *O. edulis*), or wild mollusks in lagoons and other marine environments (Robertson 2007; Aksoy et al. 2014; Coupe et al. 2018), and even those cultivated in Class A waters, for which no depuration treatment is required (Reg. EC 2285/2015; Giangaspero et al. 2009; Putignani et al. 2012).

Detection of these protozoan parasites in mollusks is of interest for public health, especially if mollusks are eaten raw or lightly cooked (Robertson 2007; Aksoy et al. 2014; Coupe et al. 2018) and is also important because they act as bio-indicators of water contamination and are thus of interest in terms of environmental health (Ghozzi et al. 2017).

Due to their high specificity and sensitivity (one oocyst per ml of hemolymph), PCR techniques have replaced microscopy tools for detecting parasitic pathogens in a complex matrix such as mollusk tissue (Miller et al. 2006). Several molecular methods, such as nested-PCR, PCR, and PCR-RFLP, have been developed to identify these protozoans (reviewed by Hohweyer et al. 2013). Other PCR methods (i.e. PCR-DGGE) have been used to detect DNA fragments of pathogens in fish and shellfish samples (El Sheikha and Montet 2016).

More recently, *q*PCR has been used to detect pathogens in mollusks (Aksoy et al. 2014; Giangaspero et al. 2014; Marangi et al. 2015), demonstrating an extremely high degree of sensitivity and specificity (up to 100%) (Hohweyer et al. 2013). However, molecular tools are currently applied to scientific research and not used in routine diagnosis, although Food and Agriculture Organization and World Health Organization guidelines support the need for tracking, monitoring and surveillance of these protozoan parasites (FAO-WHO 2014b).

At present, the molecular diagnostic industry is actively involved in developing simple and cost-effective platforms to use in pathogen detection (Biava et al. 2018). Driven by the demand for rapid and easy detection, lab-on-chip (LOC) technology has developed rapidly because multiple laboratory processes can be integrated in a miniaturized device (Yoon and Kim 2012; Marziliano et al. 2015). In the EU (Reg. EC 178/2002), traceability of all foods, including bivalve mollusks, is mandatory due to its importance in achieving food safety objectives, and a rapid tool for the detection of hazards in foods would help to prevent contaminated foods reaching consumers.

Fig. 1 Q3 platform: the Q3 disposable lab-on-chip cartridge is the core of the system, and is where the Real-Time PCR reactions occur. The Q3 Reader instrument is on the right. Both components are very compact, as shown by comparison with the coin. Dedicated software run on a PC completes the platform

The Q3 system is a small, compact LOC platform (14 × 7 × 8.5 cm, 300 g), which was developed by STMicroelectronics. Rapid and easy-to-use, it enables the amplification and detection of nucleic acids by Real-Time PCR (RT-PCR) in under 30 min (Marziliano et al. 2015; Biava et al. 2018). It has three main components (Fig. 1): the Q3 LOC Disposable Unit (DU or cartridge) where the RT-PCR reactions take place, the Q3 Reader, and Q3 software. The Q3 Reader is an electrical optical instrument, which acts as a mechanical holder of the DU, controls the thermal process on the DU, and excites and reads the optical fluorescent signals.

This study aimed to evaluate the efficiency of the Q3 system on RT-PCR protocols developed in our laboratories (Marangi et al. 2015) for detecting *Giardia*, *Cryptosporidium* and *Toxoplasma* in bivalve mollusks cultivated for human consumption.

Materials and methods

Aquarium set up and efficiency assessment

An aquarium system (Acqua and Co SRL, Cadelbosco Sopra, Ravenna, Italy)[1] of nine tanks was installed at the

[1] Aquarium system (Acqua and Co. S.r.l., Cadelbosco Sopra, Ravenna, Italy). Tanks with independent hydraulics, cool chiller module units with 0.5cv power each; 2 high-frequency 55 W UVC lamps with two spare lamps; hydraulics complete with PVC pipes, valves, fittings and discharges—double single-phase electric panel with magnetic circuit breakers according to EEC standards; 1500 L salt water tank in bins c680 with lid and module filling system.

Bonassisa Lab, Foggia, Italy. Each tank contained 20 L of water with NaCl and water conditions (salinity 34 g/L; water temperature 21 °C; oxygenation 7–8 mg/L) and room temperature (15 °C) monitored daily.

Protozoan strains

Giardia duodenalis trophozoites (strain ATCC 30957) were purchased from LGC Standards S.r.l.—Italy Office, Milan, Italy and cultured in vitro to obtain cysts, using a medium of TYI-S-33 plus 10% bovine serum and 10 mg/ml bovine bile at pH 7.8 (Keister 1983).

The maximal number of trophozoites was obtained by growing them to confluence. The culture medium containing dead trophozoites unattached to the walls was removed, and the encystation medium was added. The tube was then inserted into a CO_2 incubator at 37 °C, and after 24 h the culture tube was placed in ice for 30 min and then centrifuged at 500×*g* for 10 min. The encystation medium was subsequently removed, the pellet was resuspended in the growth medium (Keister's Modified TYI-S-33 *Giardia* Medium [ATCC Medium 2695]), and the culture tube was inserted again into the CO_2 incubator at 37 °C. After 24 h, the culture tube was placed in ice for 30 min and then centrifuged at 500×*g* for 10 min. The growth medium was subsequently removed, and the pellet containing the cysts was washed twice with PBS. To obtain a pure cyst suspension, the dead trophozoites were removed through hypotonic lysis in distilled water for 12–24 h at 4 °C, followed by subsequent washings in distilled water to remove debris (Kane et al. 1991). The hypotonic lysis cysts were then subjected to a first quality control, either by optical microscopy or direct immunofluorescence, using the Merifluor *Cryptosporidium/Giardia* (Meridian Bioscience) kit, according to the manufacturer's instructions.

Giardia (8–12 µm × 7–10 µm) fluorescent green apple structures were identified, and the number of cysts was recorded. Approximately 5×10^6 cysts were obtained from one single in vitro culture step.

Cryptosporidium parvum oocysts (at a dose of 1×10^8) were provided by the laboratory of Creative Science Company, Pentlands Science Park, Edinburgh (UK).

Toxoplasma gondii oocysts (at a dose of 6×10^6) were provided by the Department of Tropical Parasitology Medical University of Gdansk (Poland) and by the Institute of Parasitology, University of Wien (Austria), and held in a 2% H_2SO_4 solution at 4 °C.

Mollusk collection, pre-evaluation of protozoan contamination, acclimatization and spiking

Before each experiment, 2000 specimens of *M. galloprovincialis*, 2000 of *R. philippinarum* and 1000 of *O. edulis* were purchased from a depuration plant in the province of Bari, and underwent depuration for 3 weeks.

For each species, a pool was randomly selected and then subjected to RT-PCRs following the procedures described in Marangi et al. (2015) in order to exclude possible natural contamination by the three pathogens investigated.

Then 500 specimens of *M. galloprovincialis*, 500 of *R. philippinarum* and 300 of *O. edulis* were placed in the tanks and acclimatized for 10 days before processing. Salinity, nitrites, oxygenation, water temperature, room temperature and mortality parameters were constantly monitored.

Each tank and each mollusk species was spiked with each quantity (e.g., 1000, 10,000, 100,000) of each pathogen (*Toxoplasma, Cryptosporidium* and *Giardia*) separately and one at a time. An unspiked control tank was included for each mollusk species and each pathogen.

For each mollusk species, 30 live and non-injured specimens for each concentration and for each pathogen, were taken from the aquarium at 24 h and 72 h post-contamination.

The 30 specimens of each mollusk species were then divided into two subpools of 15. Hemolymph (H) and intervalvular liquid (IL) were aspirated individually from each subset of mollusks, using a needle inserted into the lateral adductor muscle; the gills (G) and digestive glands (DG) were removed following the procedures previously described (Graczyk et al. 1999) and then pooled. Four aliquots (H, IL, G, DG) were created for each pool, corresponding to the four anatomic sites of each mollusk species. All aliquots were stored at − 20 °C pending molecular analysis.

The experiments were conducted sequentially, each time using one mollusk species and one protozoan parasite species. The tanks were washed and refilled with clean water for each experiment.

DNA extraction and Real-Time PCR protocols

The Nucleospin Tissue Kit (Macherey-Nagel, Germany) was used according to the manufacturer's instructions, to extract DNA from all aliquots according to anatomic sites, mollusk species, pathogen (at all concentrations), and at 24 h and 72 h post-contamination.

RT-PCR protocols were set up based on EvaGreen® for *Toxoplasma* and *Cryptosporidium,* and TaqMan for *Giardia*. The specificity of each primer-pair was first tested with DNA of all positive controls for *Toxoplasma*, *Giardia* and *Cryptosporidium* and then with DNA of mussels used as a negative control. Each primer-pair released a fluorescent signal specific for the investigated species and genes.

For *G. duodenalis,* standard RT-PCR was performed using a CFX-96 Real-Time PCR system (BioRad Laboratories, Hercules CA, USA). Briefly, the reaction mixture

(25 µl) contained 10 µL of iQ™ Supermix (Bio-Rad Laboratories, Hercules CA, USA), 0.24 µM of TaqMan® TAMRA™ probe (FAM-5′-CCCGCGGCGGTCCCTGC TAG-3′-TAMRA) (Applied Biosystems, UK) and 3.12 µM of species-specific primers targeting SSU Rrna gene (*Giardia*-80F 5′-GACGGCTCAGGACAACGGTT-3′ and *Giardia*-127R 5′-TTGCCAGCGGTGTCCG-3′) to amplify a 62-bp fragment (Nazeer et al. 2013). The PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 42 cycles at 95 °C for 30 s, and 1 min at 62 °C. Extracted genomic DNA, the positive control for *Giardia,* and the no template (negative control, NTC, ultrapure Millipore water) in 5 µL were added to each reaction mix. Each sample was run in duplicate, and the amplification cycle threshold (*Ct*) mean value was recorded.

For *Cryptosporidium* and *Toxoplasma,* RT-PCR was performed using a CFX-96 Real-Time PCR system (BioRad Laboratories, Hercules CA, USA). Briefly, PCRs were carried out in a final volume of 20 µL, using 10 µL of SsoFast™EvaGreen® Supermix (cat. no. 172-5201; Bio-Rad Laboratories, Hercules CA, USA) and 0.5 µM of species-specific primers for COWP *C. parvum* gene (CRYINT2D-F: 5′-TTTGTTGAAGARGGAAATAGATG TG-3′ and CRY2D-R: 5′-GGACKGAAATRCAGGCATT ATCYTG-3′) or B1 *T. gondii* locus (TOXB41-F: 5′-CG AAGCTGAGATGCTCAAAGTC-3′ and TOXB169-R: 5′-AATCCACGTCTGGGAAGAACTC-3′) (see Aksoy et al. 2014; Marangi et al. 2015). Extracted genomic DNA, the positive control for *Cryptosporidium* and *Toxoplasma,* and the no template (negative control, NTC, ultrapure Millipore water) in 5 µL were added to each reaction mix. PCR cycling conditions were as follows: initial denaturation at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s, and 15 s at 50 °C (*Cryptosporidium*) or 62 °C (*T. gondii*). Melting analysis was performed at the end of each PCR run (70 °C to 95 °C at 0.5 °C/5 s). Each sample was run in duplicate, and the amplification cycle threshold (*Ct*) and melting temperature (*Tm*) mean values were calculated. The criteria used to define a positive sample were (a) a detectable amplification curve, (b) a *Tm* value equal to the *Tm* value of the specific positive control, and (c) a–dF/dT fluorescence value > 2.

Sample analysis by Q3 platform

All samples tested by standard RT-PCR were also tested by Q3. For Q3 assay, the reaction mixture consisted of 5 µL of mix (containing 2.5 µL of SsoFast™EvaGreen® Supermix (2X), 1 µL of species-specific primers (0.5 µM) for *Toxoplasma* and *Cryptosporidium* and 0.5 µL of DEPC water, or 2.5 µL of iQ™ Supermix (2X), 1 µL of TaqMan probe (3.12 µM), 0.5 µL of primers for *Giardia* and 0.5 µL of DEPC water), + 2 µL of extracted DNA sample added in each cartridge well.

A positive control (DNA extracted from *T. gondii, Cryptosporidium* and *Giardia* culture) and a negative control (water) were also included in each cartridge. The reaction parameters were as follows: initial denaturation at 98 °C for 120 s, followed by 40 cycles at 98 °C for 5 s, and 62 °C for 15 s (*Toxoplasma*); 97 °C for 180 s followed by 42 cycles at 97 ° C for 30 s and 62 °C for 60 s (*Giardia*); 98 °C for 120 s followed by 40 cycles at 98 °C for 5 s and 55 °C for 15 s (*Cryptosporidium*). Each sample was tested in triplicate.

Statistical analysis

Sensitivity (S), specificity (SP), prevalence (P), positive and negative predictive values (PV +/−) were calculated for each PCR (RT-PCR and Q3) assay, according to mollusk species, pathogen, and at 24 h and 72 h post-contamination.

The concordance between the two molecular tests used was evaluated using the kappa (*K*) coefficient (Landis and Koch 1977). *K* value varies from 0 (perfect discordance) to 1 (perfect concordance) and its interpretation was given as follows: 0.81–1.00, *very good concordance*; 0.61–0.80, *substantial concordance*; 0.41–0.60, *discrete concordance*; 0.20–0.40, *moderate concordance*; 0.10–0.20, *weak concordance* (Landis and Koch 1977).

The differences in the performance of RT-PCR and Q3 for the various anatomic sites (hemolymph, intervalvular liquid, gills, and digestive gland), compared to the initial cyst/oocyst number and in relation to the two groups of mussels/clams (repeatability) were tested by Chi Square or by Fisher's exact test, as appropriate. The software programs used were SPSS for Windows version 13.0 and WinEpi 2.0 (available online at: http://www.winepi.net/uk/index.htm).

Results

All the randomly pooled samples tested for possible natural contamination by *Toxoplasma*, *Cryptosporidium* and *Giardia* tested negative for the three investigated pathogens (data not shown).

Both RT-PCR and the Q3 platform were used to test a total of 552 pooled samples from the four anatomic sites of each mollusk species (n = 144 for *M. galloprovincialis,* n = 144 for *R. philippinarum* and n = 120 for *O. edulis*) spiked with the three pathogens at all concentrations (1000, 10,000 and 100,000 oo/cysts) at 24 h and 72 h post-spiking, together with 144 negative controls. The raw number of positive (with the corresponding *Ct* values) and negative

pool samples obtained is shown in Supplementary Table 1, and the results are summarized in Table 1. The negative controls were always negative, with both standard RT-PCR and Q3. Thus, in the absence of false positive results, the specificity and positive predictive values were 100% in all cases and are not further reported in the text and tables. In general, both RT-PCR and the Q3 system detected the three pathogens in all the mollusks species, although RT-PCR performance was better than the Q3 system, both at 24 h (20.2% vs 10.5%, respectively; $p < 0.0001$) and at 72 h (12.3% vs 5%, respectively; $p < 0.0001$) (Table 1). However, performances differed according to the protozoan species.

For *M. galloprovincialis*, the prevalence, sensitivity and negative predictive values of standard RT-PCR and the Q3 platform at 24 h and 72 h for *Toxoplasma*, *Cryptosporidium* and *Giardia* are shown in Table 2. The performance of the Q3 platform was comparable to RT-PCR in detecting *Toxoplasma* oocysts at 24 h (41.7% vs 33.3%, respectively) and 72 h (both 16.7%), while only Q3 was able to detect *Cryptosporidium* oocysts (25% vs 0%; $p = 0.0219$) at 24 h (Table 2). RT-PCR was superior to Q3 in detecting *Giardia* cysts both at 24 h (33.3% vs 8.3%) and at 72 h (12.5% vs 0%), although these differences were not statistically significant (Table 2). The two techniques performed equally well in relation to the different number of parasites spiked, detecting *Toxoplasma* at the three concentrations at 24 h, but only at the highest concentration at 72 h. For *Giardia* only RT-PCR was able to detect cysts at all three concentrations at 24 h (Supplementary Table 1). The highest positivity for the three protozoa was detected in the digestive glands (Supplementary Table 1).

RT-PCR and Q3 amplification curves of digestive glands at the concentration of 100,000, digestive glands at the concentration of 10,000, intervalvular liquid and digestive glands at the concentration of 1000 of *M. galloprovincialis* samples positive to *Toxoplasma* at 24 h post-contamination are reported in Supplementary Figs. 1 and 2, respectively.

For *R. philippinarum*, the prevalence/sensitivity and the positive predictive values of RT-PCR and Q3 at 24 h and 72 h for *Toxoplasma*, *Cryptosporidium* and *Giardia* are shown in Table 3.

RT-PCR detected more positives to *Toxoplasma* than Q3 at 24 h (79.2% vs 25%; $p < 0.0001$). No significant differences in positivity among the tissues were detected for RT-PCR and Q3. There were also no significant differences in relation to concentrations, except that RT-PCR performed better than Q3 at 72 h at the concentration of 100,000 *Cryptosporidium* oocysts.

The RT-PCR and Q3 amplification curves of hemolymph at the concentration of 100,000, gills and digestive glands at the concentration of 10,000 and intervalvular liquid at the concentration of 1000 of *R. philippinarum* samples positive to *Toxoplasma* at 24 h post-contamination are reported in Supplementary Figs. 3 and 4, respectively.

For *O. edulis,* the prevalence, sensitivity and the positive predictive values of RT-PCR and Q3 at 24 h and 72 h are shown only for *Toxoplasma* and *Cryptosporidium* (Table 4).

Giardia in *O. edulis* could not be investigated due to technical problems. RT-PCR performed better than Q3 in detecting *Toxoplasma* (100% vs 58.3%, respectively, $p < 0.001$) and *Cryptosporidium* (33.3% vs 0%; $p < 0.005$) at 24 h. Concerning concentration, both techniques detected *Toxoplasma* oocysts only at the highest concentration (100,000 oocysts) (Supplementary Table 1).

The RT-PCR and Q3 amplification curves of digestive gland and intervalvular liquid at the concentration of 100,000, intervalvular liquid and digestive glands at the concentration of 10,000, digestive glands, intervalvular liquid and gills at the concentration of 1000 of *O. edulis* samples positive to *Toxoplasma* at 24 h post-contamination are reported in Supplementary Figs. 5 and 6, respectively.

On the whole, for *M. galloprovincialis*, a *very good concordance* was found between RT-PCR and Q3 for *Toxoplasma* at 24 h and 72 h. For *R. philippinarum*, a *very good concordance* was found between RT-PCR and Q3 for *Giardia* at 24 h, and a *substantial concordance* was found for *Toxoplasma* at 72 h. For *O. edulis*, a *discrete concordance* was found between RT-PCR and Q3 for *Toxoplasma* at 24 h and a *very good concordance* was found for *Toxoplasma* at 72 h (Table 5).

Discussion

The efficiency of the Q3 "lab-on-chip" molecular platform developed by STMicroelectronics for detection of the investigated zoonotic protozoans (*Toxoplasma*, *Cryptosporidium* and *Giardia*) in three mollusk species (*M. galloprovincialis*, *R. philippinarum* and *O. edulis*) was good in comparison with the results obtained by RT-PCR. This is the first study on the use of lab-on-chip prototype technology in a food matrix, i.e., edible marine mollusks.

We expected to find a lower number of samples contaminated by spiked oo/cysts than the number of tested pools (Table 1), since laboratory conditions simulate the situation in the natural environment, i.e., not all mollusks assume protozoan parasites. Although many pools tested negative because of the very low concentration, Q3 demonstrated a good ability to detect all the tested pathogens (*Ct* ranged from 24.5 to 38), which was comparable to standard RT-PCR procedures (*Ct* ranged from 30.4 to 38); Q3 was particularly efficient for *Toxoplasma* in mussels (*Ct* ranged from 31 to 36), and in oysters (*Ct* ranged from 24.5

Table 1 Overall number and percentage of pooled tested samples of *Mytilus galloprovincialis, Ruditapes philippinarum* and *Ostrea edulis* found positive in standard Real-Time PCR (RT) and Q3 platform (Q3) at 24 h and 72 h post-contamination with all concentrations (1000, 10,000 and 100,000) for *Toxoplasma*, *Cryptosporidium* and *Giardia*

Mollusk species and Protozoans	N. of positive pools (%)					
	24 h		72 h		Total (24 h + 72 h)	
	RT (n = 24)	Q3 (n = 24)	RT (n = 24)	Q3 (n = 24)	RT (n = 48)	Q3 (n = 48)
Mytilus galloprovincialis						
Toxoplasma	8 (33.3)	10 (41.7)	4 (16.7)	4 (16.7)	12 (25.0)	14 (29.2)
Cryptosporidium	0	6 (25)	0	0	0	6 (12.5)
Giardia	8 (33.3)	2 (8.3)	3 (12.5)	0	11 (22.9)	2 (4.2)
Negative control	–	–	–	–		
Ruditapes philippinarum						
Toxoplasma	19 (79.2)[a]	6 (25)[a]	8 (33.3)	5 (20.8)	27 (56.3)[b]	11 (22.9)[b]
Cryptosporidium	8 (33.3)	2 (8.3)	3 (12.5)	0	11 (22.9)	2 (4.2)
Giardia	12 (50)	10 (41.7)	3 (12.5)	0	15 (31.3)	10 (20.8)
Negative control	–	–	–	–		
Ostrea edulis						
Toxoplasma	24 (100)[d]	14 (58.3)[d]	4 (16.7)	4 (16.7)	28 (58.3)	18 (37.5)
Cryptosporidium	8 (33.3)	0	9 (37.5)	2 (8.3)	17 (35.4)[e]	2 (4.2)[e]
Giardia	NP	NP	NP	NP	NP	NP
Negative control	–	–	–	–		
Total	87/192[f] (45.3)	50/192[f] (26.0)	34/192 (17.7)	15/192 (7.8)	121/384[c] (31.5)	65/384[c] (16.9)

NP not performed

[a–f]Statistical significance is marked with the same letters for $p < 0.01$

Table 2 Prevalence (P), sensitivity (S), negative predictive value (PV−) and 95% confidence interval in brackets of standard Real-Time PCR (RT) and Q3 platform (Q3) at 24 h and 72 h at all concentrations (1000, 10,000 and 100,000) and combination of the results for *Toxoplasma*, *Cryptosporidium* and *Giardia* in *Mytilus galloprovincialis*

PCR	*Toxoplasma*		*Cryptosporidium*		*Giardia*	
	P/S[a]	PV−	P/S	PV−	P/S	PV−
RT 24 h	33.3% (14.5–52.2)	60.0% (44.8–75.2)	0%	50.0% (35.9–64.1)	33.3% (14.5–52.2)	60.0% (44.8–75.2)
Q3 24 h	41.7% (21.9–61.4)	63.2% (47.8–78.5)	25.0% (7.7–42.3)	57.1% (42.2–72.1)	8.3% (− 2.7 to 19.4)	52.2% (37.7–66.6)
RT 72 h	16.7% (1.8–31.6)	54.5% (39.8–69.3)	0%	50.0% (35.9–64.1)	12.5% (− 0.7 to 25.7)	53.3% (38.8–67.9)
Q3 72 h	16.7% (1.8–31.6)	54.5% (39.8–69.3)	0%	50.0% (35.9–64.1)	0%	50.0% (35.9–64.1)
RT 24 h + 72 h	25.0% (7.7–42.3)	57.1% (42.2–72.1)	0%	50.0% (35.9–64.1)	22.9% (11.0–34.8)	56.5% (45.9–67.0)
Q3 24 h + 72 h	29.2% (16.3–42.0)	58.5% (47.9–69.2)	12.5% (− 0.7–25.7)	53.3% (38.8–67.9)	4.2% (− 1.5 to 9.8)	51.1% (41.0–61.2%)

[a]Prevalence coincides with sensitivity because no false positives were detected

to 35.5) compared to RT-PCR (*Ct* ranged from 34 to 38 in mussels and 30.4 to 37.8 in oysters), especially at the highest protozoan concentration and after a longer exposure time.

While RT-PCR and Q3 were found to be highly efficient in detecting *Toxoplasma*, their lower efficiency for *Giardia* and *Cryptosporidium* might be due to the gene portions used. Unlike concentration and exposure time, which obviously increase the chances of detecting the pathogens, the reasons for a higher correlation between pathogens and mollusk species are unknown.

The lack of differences between the anatomic sites—independently of the species—is a very interesting point both in terms of laboratory practice and of platform

Table 3 Prevalence (P), sensitivity (S), negative predictive value (PV−) and 95% confidence interval in brackets of standard Real Time PCR (RT) and Q3 platform (Q3) at 24 h and 72 h at all concentrations (1000, 10,000 and 100,000) and combination of the results for *Toxoplasma*, *Cryptosporidium* and *Giardia* in *Ruditapes philippinarum*

PCR	*Toxoplasma*		*Cryptosporidium*		*Giardia*	
	P/S[a]	PV−	P/S	PV−	P/S	PV−
RT 24 h	79.2% (62.9–95.4)	82.8% (69.0–96.5)	33.3% (14.5–52.2)	60.0% (44.8–75.2)	50.0% (30.0–70.0)	66.7% (51.3–82.1)
Q3 24 h	25.0% (7.7–42.3)	57.1% (42.2–72.1)	8.3% (2.7–19.4)	52.2% (37.7–66.6)	41.7% (21.9–61.4)	63.2% (47.8–78.5)
RT 72 h	33.3% (14.5–52.2)	60.0% (44.8–75.2)	12.5% (− 0.7 to 25.7)	53.3% (38.8–67.9)	12.5% (− 0.7 to 25.7)	53.3% (38.8–67.9)
Q3 72 h	20.8% (4.6–37.1)	55.8% (41.0–70.7)	0%	50.0% (35.9–64.1)	0%	50.0% (35.9–64.1)
RT 24 h + 72 h	56.3% (42.2–70.3)	69.6% (58.7–80.4)	22.9% (11.0–34.8)	56.5% (45.9–67.0)	31.3% (18.1–44.4)	59.3% (48.6–70.0)
Q3 24 h + 72 h	22.9% (11.0–34.8)	56.5% (45.9–67.0)	4.2% (− 1.5 to 9.8)	51.1% (41.0–61.2%)	20.8% (9.3–32.3)	55.8% (45.3–66.3)

[a]Prevalence coincides with the sensitivity because no false positives were detected

Table 4 Prevalence (P), sensitivity (S), negative predictive value (PV−) and 95% confidence interval in brackets of standard Real-Time PCR (RT) and Q3 platform (Q3) at 24 h and 72 h at all concentrations (1000, 10,000 and 100,000) and combination of the results for *Toxoplasma* and *Cryptosporidium* in *Ostrea edulis*

PCR	*Toxoplasma*		*Cryptosporidium*	
	P/S[a]	PV−	P/S[a]	PV−
RT 24 h	100%	100%	33.3% (14.5–52.2)	60.0% (44.8–75.2)
Q3 24 h	58.3% (38.6–78.1)	70.6% (55.3–85.9)	0%	50.0% (35.9–64.1)
RT 72 h	16.7% (1.8–31.6)	54.5% (39.8–69.3)	37.5% (18.1–56.9)	61.5% (46.3–76.8)
Q3 72 h	16.7% (1.8–31.6)	54.5% (39.8–69.3)	8.3% (− 2.7 to 19.4)	52.2% (37.7–66.6)
RT 24 h + 72 h	58.3% (38.6–78.1)	70.6% (55.3–85.9)	35.4% (21.9–48.9)	60.8% (50.0–71.5)
Q3 24 h + 72 h	37.5% (18.1–56.9)	61.5% (46.3–76.8)	4.2% (− 1.5 to 9.8)	51.1% (41.0–61.2%)

[a]Prevalence coincides with sensitivity because no false positives were detected

applicability, since it means that whole mollusks can be used for pathogen detection.

Although few documented cases of infection are actually linked to the consumption of mollusks (mostly oysters contaminated by *Giardia* and/or *Cryptosporidium*) (Baumgartner et al. 2000; Potasman et al. 2002; Robertson 2007), the lack of epidemiological information for shellfish is likely related to inadequate diagnosis, detection or reporting, due to the long time lapse between contamination and the onset of clinical signs (usually 1–2 weeks for both *Giardia* and *Cryptosporidium* infections). Consequently, medical practitioners are unlikely to associate these infections with mollusks eaten several days previously, particularly because shellfish are not commonly recognized as potential sources of infection involving these parasites (Ryan et al. 2016). Even if the cause is actually suspected, the food item has already been consumed in most cases and is therefore unavailable for testing (Robertson 2007). The recent conclusion of Sutthikornchai et al. (2016) that oysters can be an effective transmission vehicle for *Cryptosporidium* oocysts, especially within 24–72 h of contamination, with viable oocysts present at up to 7 days post-contamination, further demonstrates the reason for concern about public health. The association

Table 5 Concordance (K) and significance (p values) of standard Real-Time PCR (RT) and Q3 platform (Q3) at 24 h and 72 h at all concentrations (1000, 10,000 and 100,000) for *Toxoplasma*, *Cryptosporidium* and *Giardia* in *Mytilus galloprovincialis, Ruditapes philippinarum* and *Ostrea edulis*

Mollusk species	PCR	*Toxoplasma*		*Cryptosporidium*		*Giardia*	
		K	*p*	K	*p*	K	*p*
Mytilus galloprovincialis	RT 24 h versus Q3 24 h	0.824	< 0.01	nc	–	0.308	< 0.05
	RT 72 h versus Q3 72 h	1.000	< 0.01	nc	–	–	–
Ruditapes philippinarum	RT 24 h versus Q3 24 h	0.161	ns	0.308	< 0.05	0.833	< 0.01
	RT 72 h versus Q3 72 h	0.690	< 0.01	nc	–	–	–
Ostrea edulis	RT 24 h versus Q3 24 h	0.651	< 0.01	nc	–	np	
	RT 72 h versus Q3 72 h	1.000	< 0.01	0.263	ns	np	–

Concordance (K) value and its interpretation: > 0.81 almost perfect concordance; 0.61–0.80 substantial concordance; 0.41–0.60 discrete concordance; 0.20–0.40 moderate concordance; 0.1–0.20 weak concordance

np not performed, *ns* not significant, *nc* not computable because one of the variables is a constant (all negatives)

between shellfish consumption and *Toxoplasma* infection is even less likely to be recognized, because immunocompetent individuals are unlikely even to recognize that they have been infected. When infection is suspected, and congenital transmission is excluded, it is more likely that water sources, undercooked meat products or direct transmission from cat feces are investigated as possible sources.

Current European legislation does not include the investigated protozoans (*Giardia*, *Cryptosporidium* and *Toxoplasma*) in the list of routinely monitored pathogens, including in mollusk species. However, according to the European Technology Platform "Food for Life"—which emphasizes the need not only to detect parasites, but also to develop specific innovative methods to include in HACCP practice—the Q3 platform might be a useful and innovative tool for screening the safety of these food products.

Conclusion

The Q3 platform can be considered a valuable molecular tool for detecting all the investigated pathogens, with a particularly high level of efficiency in detecting *Toxoplasma.* Q3 detection efficiency for *Cryptosporidium* could be improved by using different target genes and/or by the use of different PCR protocols.

This work represents a first technological approach and a challenge for implementing pathogen detection in different mollusk species. Pending improvements to its performance (even at a lower range of pathogen concentration) on all mollusk species, the "lab-on-chip" molecular integrated Q3 platform provides a feasible tool that is small, rapid and easy-to-use, and thus suitable for in-the-field testing by companies producing and/or marketing mollusks.

Acknowledgements The study was funded by "New Strategies for Improvement of Food Safety: Prevention, Control, Correction" (S.I.Mi.S.A.)—PON02_00186_3417512—PON Ricerca e Competitività 2007–2013" (PO Puglia FESR 2007e2013 Asse I, Linea 1.2dPO Puglia FSE 2007e2013 Asse IV). The authors wish to thank Anna Lass of the Department of Tropical Parasitology, Medical University of Gdansk (Poland), and Anja Joachim of the Institute of Parasitology, University of Wien (Austria) for providing *Toxoplasma* oocyst strains, Tiziana Caradonna for her helpful work in the lab, Tommaso Marazia for his outstanding assistance in aquarium system management, and Alessandra Barlaam and Sarah Christopher for the English revision of the MS.

Authors' contributions AG, GN and DO conceived the study design; LP performed in vitro culture; FF and MC developed the Q3 system and trained for the Q3 analysis; MM, MSL and GA collected samples and performed the Real Time and Q3 analysis; GC performed the statistical analysis; AG, GN, MM, LP, MC and DO interpreted the data and wrote the paper. All authors contributed to editing the manuscript. All authors read and approved the final version of the manuscript.

References

Aksoy U, Marangi M, Papini R, Ozkoc S, Bayram Delibas S, Giangaspero A (2014) Detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* in *Mytilus galloprovincialis* from Izmir Province coast (Turkey) by Real time PCR/High-Resolution Melting analysis (HRM). Food Microbiol 44:128–135

Baumgartner A, Marder HP, Munzinger J, Siegrist HH (2000) Frequency of *Cryptosporidium* spp. as cause of human gastrointestinal disease in Switzerland and possible sources of infection. Schweiz Med Wochenschr 130:1252–1258

Biava M, Colavita F, Marzorati A, Russo D, Pirola D, Cocci A, Petrocelli A, Delli Guanti M, Cataldi G, Kamara TA, Kamara AS, Konneh K, Cannas A, Coen S, Quartu S, Meschi S, Valli MB, Mazzarelli A, Venditti C, Grassi G, Rozera G, Castilletti C, Mirazimi A, Capobianchi MR, Ippolito G, Miccio R, Di Caro A (2018) Evaluation of a rapid and sensitive RT-qPCR assay for the detection of Ebola Virus. J Virol Methods 252:70–74

Coupe A, Howe L, Burrows E, Sine A, Pita A, Velathanthiri N, Vallée E, Hayman D, Shapiro K, Roe WD (2018) First report of Toxoplasma gondii sporulated oocysts and Giardia duodenalis in

commercial green-lipped mussels Perna canaliculus in New Zealand. Parasitol Res 117:1453–1463

Dubey JP, Jones JL (2008) *Toxoplasma gondii* infection in humans and animals in the United States. Int J Parasitol 38:1257–1278

El Sheikha AF, Montet D (2016) How to determine the geographical origin of seafood? Crit Rev Food Sci Nutr 56:306–317

Eurostat (2015) http://ec.europa.eu/eurostat/statistics-explained/index.php/Aquaculture_statistics/. Accessed 13 Dec 2018

FAO (2015) Fisheries and aquaculture statistics http://www.fao.org/3/a-i7989t.pdf/. Accessed 13 Dec 2018

FAO-WHO (2014a) http://apps.who.int/iris/bitstream/10665/112672/1/9789241564700_eng.pdf/. Accessed 13 Dec 2018

FAO-WHO (2014b) http://www.fao.org/tempref/codex/Meetings/CCFFP/ccffp34/Shellfish%20Sanitation%20Initiation%20meeting%20Report%20%20final%20for%20CCFFP.pdf. Accessed 13 Dec 2018

Giangaspero A, Cirillo R, Lacasella V, Lonigro A, Marangi M, Cavallo P, Berrilli F, Di Cave D, Brandonisio O (2009) *Giardia* and *Cryptosporidium* in inflowing water and harvested shellfish in a lagoon in Southern Italy. Parasitol Int 58:12–17

Giangaspero A, Papini R, Marangi M, Koehler AV, Gasser RB (2014) *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int J Food Microbiol 171:62–67

Graczyk TK, Fayer R, Lewis EJ, Trout JM, Farley CA (1999) *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol Res 85:518–521

Ghozzi K, Marangi M, Papini R, Lahmar I, Challouf R, Houas N, Ben Dhiab R, Normanno G, Babba H, Giangaspero A (2017) First report of Tunisian coastal water contamination by protozoan parasites using mollusk bivalves as biological indicators. Maar Pollut Bull 117:197–202

Hohweyer J, Dumètre A, Aubert D, Azas N, Villena I (2013) Tools and methods for detecting and characterizing *Giardia, Cryptosporidium* and *Toxoplasma* parasites in marine mollusks. J Food Prot 76:1649–1657

Kane AV, Ward HD, Keusch GT, Pereira ME (1991) In vitro encystation of *Giardia lamblia*: large-scale production of in vitro cysts and strain and clone differences in encystation efficiency. J Parasitol 77:974–981

Keister DB (1983) Axenic culture of *Giardia lamb*lia in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg 77:487–488

Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33(1):159–174

Marangi M, Giangaspero A, Lacasella V, Lonigro A, Gasser RB (2015) Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. Mol Cell Probes 29:122–125

Marziliano N, Notarangelo MF, Cereda M, Caporale V, Coppini L, Demola MA, Guidorossi A, Crocamo A, Pigazzani F, Boffetti F, Del Giudice F, Orsini F, Pirola D, Cocci A, Manzalini C, Casu G, Bianchessi M, Ardissino D, Merlini PA (2015) Rapid and portable, lab-on-chip, point-of-care genotyping for evaluating clopidogrel metabolism. Clin Chim Acta 451:240–246

Miller WA, Gardner IA, Atwill ER, Leutenegger CM, Miller MA, Hedrick RP, Melli AC, Barnes NM, Conrad PA (2006) Evaluation of methods for improved detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). J Microbiol Methods 65:367–379

Mmbaga BT, Houpt ER (2017) *Cryptosporidium* and *Giardia* infections in children: a review. Pediatr Clin North Am 64:837–850

Nazeer JT, Khalifa KES, von Thien H, El-Sibaei MM, Abdel-Hamid MY, Tawfik RA, Tannich E (2013) Use of multiplex real-time PCR for detection of common diarrhea causing protozoan parasites in Egypt. Parasitol Res 112:595–601

Potasman I, Paz A, Odeh M (2002) Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clin Infect Dis 35:921–928

Putignani L, Mancinelli L, Del Chierico F, Menichella D, Adlerstein D, Angelici MC, Marangi M, Berrilli F, Caffara M, di Regalbono DA, Giangaspero A (2012) Investigation of *Toxoplasma gondii* presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). Exp Parasitol 127:409–417

Robert-Gangneux F, Dardé ML (2012) Epidemiology of and diagnostic strategies for toxoplasmosis. J Clin Microbiol 25:264–296

Robertson LJ (2007) The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: a review. Int J Food Microbiol 120:201–216

Ryan U, Zahedi A, Paparini A (2016) *Cryptosporidium* in humans and animals a one health approach to prophylaxis. Parasite Immunol 38:535–547

Sutthikornchai C, Popruk S, Chumpolbanchorn K, Sukhumavasi W, Sukthana Y (2016) Oyster is an effective transmission vehicle for *Cryptosporidium* infection in human. Asian Pac J Trop Dis 9:562–566

Thompson RC, Olson ME, Zhu G, Enomoto S, Abrahamsen MS, Hijjawi NS (2005) *Cryptosporidium* and cryptosporidiosis. Adv Parasitol 59:77–158

Yoon JY, Kim B (2012) Lab-on-a-chip pathogen sensors for food safety. Sensors (Basel) 12:10713–10741