A method for the detection of *Cryptosporidium parvum* in apple juice based on microfiltration and real-time polymerase chain reaction

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Summary

A method for the detection of *Cryptosporidium parvum* oocysts in apple juice was developed. The method employs separation of oocysts by microfiltration, DNA isolation by lysis coupled to chaotropic solid phase extraction and single-tube nested real-time polymerase chain reaction. The detection limit of the method was 1×10^1 *C. parvum* oocysts per 250 ml of apple juice. Thanks to its sensitivity, the method may be useful for improved control of the contamination of apple juice samples by *C. parvum* oocysts.

Keywords

Cryptosporidium parvum; microfiltration; DNA; polymerase chain reaction

The protozoon *Cryptosporidium parvum* is a pathogenic parasite that causes epidemies mainly via contaminated water [1, 2]. Food may also be contaminated by *C. parvum* oocysts and cause infection [3, 4]. A possible vehicle of *C. parvum* oocysts may be unpasteurized apple juice (also called cider in USA or Great Britain) [5, 6]. The beverage may be contaminated if produced from contaminated apples.

Apple juice may be contaminated with *C. parvum* oocysts at low levels, which have however a potential to cause the infection. Available analytical methods face sensitivity problems with detection of such low levels of *C. parvum* oocysts. The most sensitive currently available analytical methods consist of oocyst separation and subsequent specific detection of DNA by polymerase chain reaction (PCR). DENG and CLIVER [7] developed a method using oocyst separation by formalin-ethylacetate fractionation or flotation in a saccharose gradient, with subsequent DNA isolation and PCR. The authors reported a detection limit of 30–100 oocysts per 100 ml of apple juice. Using the same saccharose gradient centrifugation coupled to more sensitive nested PCR, GARCIA et al. [8] achieved a detection limit of 10 *C. parvum* oocysts per 50 ml of apple juice. Another method was developed by FRAZAR and ORLANDI [9] who used immunomagnetic separation with subsequent nested PCR and achieved a detection limit of 50 *C. parvum* oocysts per 10 ml of apple juice.

In this study, we attempted to improve the sensitivity of the detection of *C. parvum* oocysts in apple juice. We made use of our preliminary experience with the development of a method for the detection of the protozoon in raw milk based on separation of the oocysts by microfiltration and detection of DNA by real-time nested PCR [10]. However, apple juice is a different matrix and several adaptations were necessary. Fresh, unpasteurized apple juice is less viscous than milk, due to absence of fat and a low content of proteins, but it contains considerable amounts of solid particles, which may cause clotting of microfilters. For this reason, separation process had to be optimized. Another problem is connected with PCR inhibitors, which are contained in the components of apple juice separated in the same fraction as *C. parvum* oocysts. This problem had to be overcome by optimizing DNA isolation.

MATERIALS AND METHODS

Parasite oocysts

Heat-inactivated *Cryptosporidium parvum* oocysts of cervine origin were obtained from Creative Science Company (Edinburgh, United King-

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dom). The oocysts were suspended in phosphate-buffered saline solution with added antibiotics (penicillin, streptomycin) at 1×10^7 oocysts per ml, and stored at 4 °C.

Artificially contaminated apple juice samples

Each 250 ml sample of 100% apple juice (100% fruit content; Dizzy, Jankowice, Poland) was mixed with 1.0 ml of the oocyst suspension in distilled water, containing 1×10^5, 1×10^4, 1×10^3, 1×10^2 , 1×10^1 and 1×10^0 oocysts, respectively. All samples were prepared in duplicate and stored at 25 °C until analysis for a maximum of 1 h.

Microfiltration

Membrane microfilter made of a mixture of cellulose acetate and cellulose nitrate (diameter, 47 mm; pore size, 3.0 μm; Advantec, Tokyo, Japan; Cat. no. A300A047A) was housed in a glass filtration apparatus (Millipore, Billerika, Massachusetts, USA; Cat. no. XX1004700 and XX1004705) and the artificially contaminated apple juice was passed through by application of vacuum. After filtering the sample, the glass chimney was rinsed with 3–5 ml of distilled water.

Elution

The filter was placed in a 50 ml Falcon-type polypropylene tube with conical bottom (Sarstedt, Nümbrecht, Germany; Cat. no. 62.547.004). A volume of 50 ml of a filter-eluting solution PET was added, containing 0.02% (w/v) sodium pyrophosphate, 0.01% (v/v) Tween 80 and 0.03% trisodium EDTA [10, 11]; all chemicals were from Merck, Darmstadt, Germany. Tubes were placed into incubator shaker (Innova 4000; New Brunswick Scientific, Edison, New Jersey, USA) and shaken at 40 °C for 30 min at 4 Hz. Afterwards, samples were centrifuged at 2300×*g* for 10 min at 25 °C, the filter was carefully removed from the tube and the suspension was centrifuged again at the same conditions. After centrifugation, the supernatant was discarded and the sediment was used for DNA isolation.

DNA isolation

Four methods were used to isolate template *C. parvum* DNA for downstream analysis by PCR. Method A was a simple chelex-based InstaGene procedure (Bio-Rad, Hercules, California, USA), used according to the manufacturer's protocol. Briefly, the sediment from the previous step was washed by resuspension in 1 ml of distilled water and centrifugation at 13000×*g* for 3 min. The obtained washed sediment was resuspended in 200 μl of InstaGene suspension and incubated at 56 °C for 3 h. Then it was incubated at 100 °C for 8 min, centrifuged at 13000×*g* for 3 min, and the supernatant was saved. Volumes of 5 μl of the supernatant were used as a template for PCR.

Method B was chaotropic solid phase extraction (SPE) using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) carried out according to the manufacturer's protocol of 2006 for Gram-positive bacteria with final elution of isolated DNA in 50 μl.

Method C consisted of lysis and of chaotropic SPE using QIAamp DNA Mini kit (Qiagen). The sediment after microfiltration was resuspended in 180 μl of lysis solution ATL (included in the kit), and the oocysts were lysed by incubation at 95 °C for 20 min. Subsequently, 20 μl of proteinase K solution (600 mAU·ml^{-1}, included in the kit) was added, and the solution was incubated at 55 °C for 3 h. The reaction was stopped by incubating at 95 °C for 10 min. Further isolation steps were performed with QIAamp DNA Mini kit according to the manufacturer's instructions of 2003. The product was eluted in a volume of 50 μl.

Method D consisted of lysis according to Method C, followed by SPE according to Method B.

DNA was isolated from duplicate samples with each concentration of oocysts.

Polymerase chain reaction

Two PCR methods were used for the detection of specific DNA. First, a modified real-time PCR of Guy et al. [12] was used. Each PCR sample (25 μl) contained 500 nmol·l^{-1} of primer CP_{COWP}_166F (caa att gat acc gtt tgt cct tct g), 500 nmol·l^{-1} of primer CP_{COWP}_316R (ggc atg tcg att cta att cag ct), 200 nmol·l^{-1} of the probe CP_{COWP}_255P (FAM-tgc cat aca ttg ttg tcc tga caa att gaa t-TAMRA; all oligonucleotides synthesized by Qiagen Operon, Cologne, Germany), 1.5 U of Cheetah Hot Start Taq DNA polymerase (Biotium, Hayward, California, USA), 200 μmol·l^{-1} of dNTP mixture with dUTP (Applied Biosystems, Foster City, California, USA), 4.0 mmol·l^{-1} $MgCl_2$, 2.5 μl of 10× concentrated PCR buffer supplied with the polymerase, 0.1 U of Uracil-DNA glycosylase, heat-labile (Roche, Mannheim, Germany) and 2.5 μl of the sample DNA solution. The temperature programme for PCR consisted of a 10 min incubation at 50 °C, initial denaturation at 95 °C for 2 min and 45 cycles (95 °C for 15 s, 60 °C for 60 s).

The second method was a highly sensitive single-tube nested real-time PCR [13]. Each PCR sample (volume 25 μl) contained 200 nmol·l^{-1} each of outer primers CPrI (aaa ccc ctt tac aag tat caa

ttc ga) and CPrII (ttc cta tgt ctg gac ctg gtg agt t), inner primers CPrF3 (cag ttg ggg gca ttt gtt tgt att) and CPrR3 (ccc cta act ttc gtt ctt gat t), and of the probe CPrP3-1 (FAM-cat cct tgg caa atg ctt tcg cat tag-TAMRA; all oligonucleotides synthesized by Qiagen Operon), 200 μmol·l^{-1} of dNTP mixture with dUTP, 3.5 mmol·l^{-1} $MgCl_2$, 1.5 U Cheetah Hot Start Taq DNA polymerase, 2.5 μl of 10× concentrated PCR buffer supplied with the polymerase, 0.5 U Uracil-DNA glycosylase, heat-labile, and 2.5 μl of the sample DNA solution. The temperature programme consisted of a 10 min incubation at 50 °C the initial denaturation at 94 °C for 2 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s and polymerization at 72 °C for 60 s, and 30 cycles of denaturation at 94 °C for 15 s, and annealing and polymerization at 53 °C for 60 s.

PCR was performed in white low-profile eight-microtube strips and the fluorescence was measured through optical caps in Opticon 2 real-time PCR cycler (MJ Research, Waltham, Massachusetts, USA) in channel 1 optimal for FAM with manual threshold setting at a fluorescence value of $10^{-1.4}$. Analyses were carried out in duplicate.

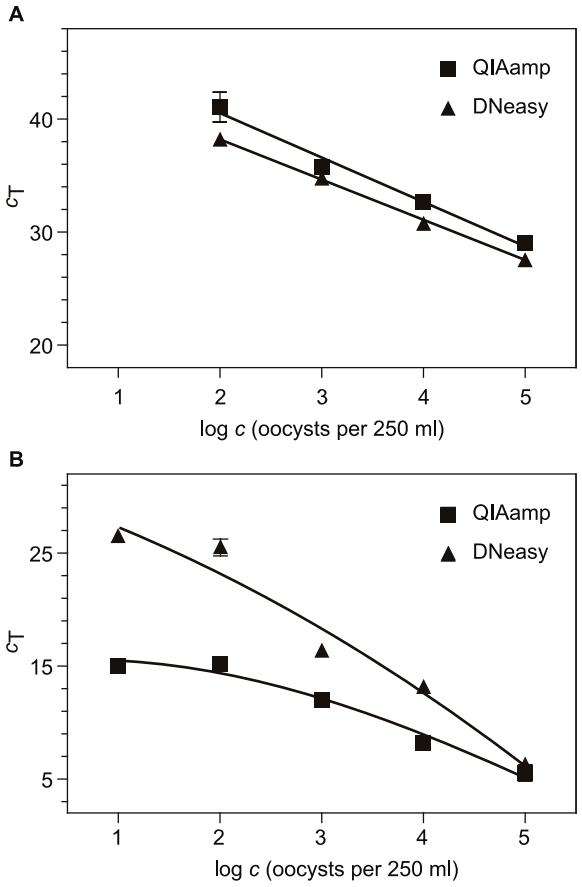

Fig. 1. Results the analysis of apple juice samples artificially contaminated with *C. parvum* oocysts by real-time PCR (A) and single-tube nested real-time PCR (B).

DNA was isolated by the methods involving lysis with subsequent chaotropic SPE using Method C (QIAamp kit; squares) and Method D (DNeasy Mini kit; triangles). Results (mean ± standard dfeviation) of duplicate analysis of duplicate samples at each level of contamination are presented. c_T - threshold cycle.

RESULTS AND DISCUSSION

In order to improve the sensitivity of the detection of *C. parvum* oocysts in apple juice, we combined various procedures for oocyst separation, DNA isolation and PCR detection. In preliminary experiments, separation of oocysts was carried out by previously published procedures – by microfiltration [10], formalin-ethylacetate fractionation [7] and flotation in a saccharose gradient [7]. DNA from the separated fractions was isolated using InstaGene formulation (DNA isolation Method A) and analysed by real-time PCR. In all cases, real-time PCR was inhibited, as demonstrated by the failure of positive controls to be amplified in the presence of DNA samples (data not shown). Such a result can be explained by the presence of PCR inhibitors, such as phenolics, in the apple juice [7, 9]. This indicated that a more effective DNA isolation was needed to be used.

In the second round of preliminary experiments, individual oocyst separation procedures were combined with SPE using DNeasy and QIAamp kits (DNA isolation methods B and C), respectively. With samples of 100 ml apple juice contaminated with 10^4 and 10^3 oocysts, respectively, only microfiltration combined with SPE using QIAamp (DNA isolation Method C) yielded positive real-time PCR results (data not shown). This indicated that formalin-ethylacetate fractionation and flotation in a saccharose gradient were not effective methods for separation of oocysts from apple juice. It was also demonstrated that effectiveness of DNA isolation in terms of removal of PCR inhibitors is crucial at the analysis of this food matrix by PCR.

In further experiments, apple juice samples of 250 ml, artificially contaminated with 10^5–10^0 *C. parvum* oocysts, were analysed using procedures consisting of microfiltration, chaotropic SPE and real-time PCR. Real-time PCR was used in a normal format or in a single-tube nested format, the latter being a more sensitive version [13, 14]. In order to improve the efficiency of DNA isola-

tion by DNeasy kit, it was supplemented by the lysis step from QIAamp (DNA isolation Method C) and the complete procedure (DNA isolation Method D) was also tested. The results showed that a detection limit of 1×10^2 oocysts per 250 ml was achieved by both DNA isolation methods with lysis (Method C and Method D), when coupled to normal real-time PCR (Fig. 1A), and a detection limit of 1×10^1 oocysts per 250 ml was achieved by both DNA isolation methods with lysis (Method C and Method D), when coupled to nested real-time PCR (Fig. 1B).

The differences in the detection of DNA isolated by the two methods by normal real-time PCR are only minor and may be taken as being within the experimental variability (Fig. 1A). On the other hand, more pronounced differences between the detection of DNA isolated by the two methods have been observed with nested real-time PCR. This PCR is, in principle, a non-quantitative method, for which non-linear response is characteristic (Fig. 1B). Although the differences in amplifiability of DNA isolated by the two methods do not lead to a different detection limit, they suggest that SPE with QIAamp (Method C) may be a more suitable procedure in this application. It is difficult to explain such a difference at the current status of knowledge, but it probably reflects the fact that QIAamp produces higher yields of partially fragmented DNA, which performs well as a template for PCR with amplicons of a small size.

The sensitivity of the method may be negatively influenced at certain apple juice samples by lower performance of microfiltration. Different apple juices may contain different amounts of solid particles, which may cause problems by clotting the microfilter and reducing the volume of juice that may be processed. With problematic apple juices, microfilters with a different pore size or diameter, or additional pre-filtration step may have to be used.

The developed method for the detection of *C. parvum* oocysts in apple juice was more sensitive, faster and less laborious than previously published methods. The method of Deng and Cliver [7] had a detection limit of 30–100 oocysts per 100 ml of apple juice. This method was also more laborious, using complicated formalin-ethylacetate sedimentation. Formalin is a toxic substance, which needs careful handling and formalin may inhibit PCR [15]. Our method was also more sensitive than that of Frazar and Orlandi [9] with a detection limit of 50 oocysts per 10 ml, and more sensitive than the method of Garcia et al. [8] with a detection limit of 10 *C. parvum* oocysts per 50 ml of apple juice.

In order to improve the reliability of the complete method, it may be supplemented with internal amplification control. For this purpose, various exogenous systems may be utilized [16, 17].

CONCLUSION

The developed method for the detection of *C. parvum* oocysts in apple juice, consisting of separation oocysts by microfiltration, DNA isolation by Method C (chaotropic SPE using QIAamp kit), and single-tube nested real-time PCR, has a detection limit of 1×10^1 oocysts per 250 ml. Thanks to its sensitivity, it may be useful for improved control of the contamination of apple juice samples by *C. parvum* oocysts.

Acknowledgements

This study was supported by Slovak Research and Development Agency, project APVT 27-044402.

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Received 9 June 2010; revised 29 July 2010; accepted 2 August 2010.