# Detection of *Cryptosporidium parvum* by polymerase chain reaction

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**Summary**

*Cryptosporidium parvum* is a protozoan parasite responsible for the outbreaks of cryptosporidiosis in humans. Several outbreaks of cryptosporidiosis associated with water and raw food occurred. Polymerase chain reaction (PCR) for direct detection of *Cryptosporidium parvum* oocysts in water, milk and apple juice was developed. Real-time PCR was able to detect $10^2$ oocysts/ml in water, milk and apple juice. The sensitivity of PCR methods was improved using nested PCR, in which second round was modified by real-time PCR. In this case, the detection limit of nested PCR was less that $10^1$ oocysts/ml of water, milk and apple juice, which compares favourably with the detection limit of the conventional PCR method - $10^3$ oocyst/ml after DNA isolation by chaotropic solid phase extraction used in both cases.

**Keywords**

*Cryptosporidium parvum*; water; milk; apple juice; polymerase chain reaction

Over recent decades, parasitic protozoa have been recognised as having great potential to cause water-borne and food-borne diseases. *Cryptosporidium* causes diarrhoea in livestock and in human. The majority of human cases of the illness are caused by *Cryptosporidium parvum* [1]. The infective oocysts may be transmitted directly by the feacal-oral route, as well as through contamination of water supplies and contaminated raw food, with humans (person-to-person contact) and other animals (particularly calves and lambs) as environmental sources. *C. parvum* oocysts are commonly found on dairy farms and may be transmitted to humans through contaminated raw milk and dairy products [2]. A few foodborne outbreaks have been linked to contaminated apple juice, fresh fruit and vegetables [1]. The oocysts are environmentally stable, surviving routine waste-water treatment, resistant to common disinfectants and water chlorine treatment is not effective [3]. As few as 10 oocysts can cause illness in infant and as few as 132 oocysts per individual have been reported as a infectious dose for healthy humans [4].

Traditionally, the detection of *C. parvum* oocysts was based on immunological tests, histochemical staining and microscopy. However, these techniques may not always identify genus and species. Recently, several molecular-biological methods have been developed for detecting the oocysts in wastewater, drinking water and food by identifying *C. parvum* nucleic acids using polymerase chain reaction (PCR) [4]. PCR has provided the basis for the development of a new generation of diagnostics. PCR has been shown to be more sensitive and more accurate than immunoassays for the detection of *Cryptosporidium* in environmental samples. Nested PCR, which involves a secondary round of amplifications using nested primers, has been applied to *Cryptosporidium* to increase both specificity and sensitivity [3]. In recent years, real-time PCR method using the TaqMan fluorogenic detection system has been developed for microorganisms in the environment [5, 6].

In this study, the rapid, sensitive methods based on polymerase chain reaction for *C. parvum* oocysts detection in water, milk and apple juice are evaluated.

## MATERIAL AND METHODS

### Source of *Cryptosporidium parvum* oocysts

*Cryptosporidium parvum* oocysts ($5.10^6$ oocysts per 1 ml) were obtained from Moredun (Penicuik,

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United Kingdom). Oocysts were supplied as a purified suspension in phosphate-buffered saline solution with added antibiotics and stored at 4 °C.

### Sample preparation

Samples of water, milk and apple juice obtained from retail markets in Slovakia were used. A volume of 500 µl of distilled water, low fat milk (Rajo, Slovakia; ultra high temperature milk with 0.1% fat) and apple juice samples (McCarter, Slovakia, 100% apple juice from concentrate) were artificially contaminated with serial 10-fold dilutions from purified oocyst suspension. Concentration of oocysts ranged from $10^5$ to $10^0$ oocysts per 1 ml of the samples.

### DNA isolation and purification

Two methods were adapted to obtain template *C. parvum* DNA for PCR. One method included isolation of DNA from oocysts with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A volume of 500 µl of oocysts suspension was centrifuged at 13700 *g* for 5 min (Biofuge Pico Heraeus, DJB Labcare, United Kingdom). The pellet was resuspended in 180 µl of ATL solution (Qiagen) and treated at 95 °C for 20 min, then digested with proteinase K (600 mAU.ml$^{-1}$, Qiagen) at 55 °C for 3 h. The reaction was stopped by heating at 95 °C for 10 min. DNA was extracted using QIAamp DNA Mini Kit columns according to manufacturer's instructions [7].

The second method was based on simple heat lysis of oocysts. The pellet of oocysts was resuspended in 100 µl of 1x buffer supplied with HotStarTaq DNA polymerase (Qiagen), incubated at 95 °C for 20 min and then directly used as a template for PCR [8].

## POLYMERASE CHAIN REACTION ASSAY

### Conventional PCR

Each reaction sample (volume 25 µl) contained 500 nmol.l$^{-1}$ of each primer SB012F and SB012R [7] and synthesized by Qiagen Operon (Cologne, Germany) amplifying 458 bp fragment. 200 µmol.l$^{-1}$ of each deoxynucleoside triphosphate (dNTP; Applied Biosystems, Foster City, California, USA), 1.5 mmol.l$^{-1}$ $MgCl_2$, 1.5 U of HotStarTaq polymerase, 2.5 µl of 10x concentrated PCR buffer supplied with the polymerase and 2.5 µl DNA sample. Reactions were performed in Multiply® - µStrip 0,2 ml chain (Sarstedt, Nümbrecht, Germany) in a GeneAmp 9700 thermal cycler (Applied Biosystems) using a programme involving the initial denaturation at 94 °C for 15 min followed by 35 cycles (denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 1 min) and then final extension at 72 °C for 10 min [7]. 12,5 µl of the PCR product were run on 1.5% agarose gels in TAE buffer (0.04 mol.l$^{-1}$ TRIS, 0.02 mol.l$^{-1}$ glacial acetic acid, 0.001 mol.l$^{-1}$ EDTA, pH 8.0), stained with ethidium bromide and viewed by ultraviolet transillumination.

### Real-time PCR (modified protocol of Fontaine and Guillot [6])

The primers and TaqMan probe used for the real-time PCR were positioned inside a specific 452 bp *C. parvum* sequence in GenBank AF188110 (National Center for Biotechnology, Bethesda, Maryland, USA). The fluorescent TaqMan probe CPrt1P labelled with 6-carboxyfluorescein and quenched with tetramethyl-6-carboxyrhodamine. The forward primer CPrt1F and reverse primer CPrt1R [6] amplified a 138 bp fragment. Primers and probe were synthesized by Qiagen Operon.

The reaction sample of PCR (volume 25 µl) contained 500 nmol.l$^{-1}$ of each primer CPrtF and CPrtR, 200 nmol.l$^{-1}$ of the probe CPrt1P, 1.5 U of HotStarTaq polymerase, 200 µmol.l$^{-1}$ of each dNTP, 4.5 m.mol$^{-1}$ $MgCl_2$, 2.5 µl of 10x concentrated PCR buffer supplied with the polymerase and 2.5 µl DNA sample. Reactions were performed in white low-profile eight-microtube strips and the fluorescence was measured through optical caps in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, Massachusetts, USA). A programme for PCR consisted of the initial denaturation at 95 °C for 15 min and 45 cycles at 95 °C for 15 s and at 60 °C for 1 min. In addition, the reaction mixture contained and internal amplification control system (Applied Biosystems) measured in channel for VIC/JOE dyes.

### Nested PCR (modification of Bialek et al. [9])

The outer primer set CPrF1 and CPrR1 is complementary to a region of small subunit ribosomal RNA gene of *Cryptosporidium parvum*, amplifying a 676 bp fragment. The inner primer set CPrF2 and CPrR2 delimits a 285-nucleotide sequence [9]. Oligonucleotides were synthesized by Qiagen Operon.

The reaction sample for the first round of PCR (volume 50 µl) contained 500 nmol.l$^{-1}$ of each outer primer CPrF1 and CPrR1, 100 µmol.l$^{-1}$ of each dNTP, 2.5 mmol.l$^{-1}$ $MgCl_2$, 1.5 U of HotStarTaq polymerase, 5 µl of 10x concentrated PCR buffer supplied with the polymerase and 10 µl DNA preparation. Reactions were performed in the GeneAmp 9700 thermal cycler using a programme involving the initial denaturation at 94 °C

for 15 min, 35 cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min) and then final extension at 72 °C for 5 min.

The reaction sample for second round of PCR was identical with the reaction sample for first round of PCR, except of the volume of 1 μl of DNA template (product of the first PCR reaction) and 500 nmol.l$^{-1}$ of each inner primer CPrF2 and CPrR2. Reactions were performed in the GeneAmp 9700 thermal cycler using a programme involving the initial denaturation at 94 °C for 15 min, 30 cycles (denaturation at 94 °C for 30 s, annealing at 68 °C for 1 min) and then final extension at 72 °C for 5 min. Annealing temperature was optimised using temperature gradient PCR. 12.5 μl of the products after first and second round of PCR were run on 1.5% agarose gels in TAE buffer, stained with ethidium bromide and viewed by ultraviolet transillumination.

**Nested PCR in a combination with real-time PCR (originally developed method)**

In general, nested PCR was developed for decreasing the detection limit of conventional PCR methods. A new system for *C. parvum* oocysts detection, combination of nested PCR and real-time PCR for second round, was developed to decrease the detection limit, to shorten the time of the analysis and to eliminate the use of electrophoresis with ethidium bromide staining.

The first round was based on nested PCR [9] and the second round was originally modified using real-time PCR. The first round of PCR utilized the outer primer set CPrF1 and CPrR1, reaction sample of PCR and the machine programme was the same as in the previously described nested PCR.

The pair of primers, the forward primer CPrF3 (5'-CAG TTG GGG GCA TTT GTT TGT ATT-3') and reverse primer CPrR3 (5'-CCC CTA ACT TTC GTT CTT GAT T-3') and the TaqMan probe CPrP3-1 labelled with 6-carboxyfluorescein and quenched with tetramethyl-6-carboxyrhodamine (5'-FAM-CAT CCT TGG CAA ATG CTT TCG CAT TAG-TAMRA-3') for second round were designed to detect *C. parvum* (accession number AF093492, GenBank; nucleotide position 828 to 945), amplifying a 118 bp fragment. The primers and the probe were synthesized by Qiagen Operon.

The reaction sample of real-time PCR (volume 50 μl) contained 500 nmol.l$^{-1}$ of each primer CPrtF3 and CPrtR3, 1.0 μmol.l$^{-1}$ of the probe CPrtP3-1, 1.5 U of HotStarTaq polymerase, 500 μmol.l$^{-1}$ of each dNTP, 3.5 mmol.l$^{-1}$ $MgCl_2$, 5.0 μl of 10x concentrated PCR buffer supplied with the polymerase and 1.0 μl of DNA product from first round of PCR. Reactions were performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector. A programme for PCR consisted of the initial denaturation at 95 °C for 15 min and 40 cycles at 95 °C for 15 s and at 53 °C for 1 min. Annealing temperature was optimised using temperature gradient PCR.

## RESULTS AND DISCUSSION

The sensitivity of the PCR protocols described above was evaluated using series of decimally diluted *C. parvum* oocyst suspension of known concentration. The results were summarised in Tab. 1.

Heat lysis, a simple DNA isolation method, was not applicable for *C. parvum* oocysts from milk and apple juice. The inhibitors from these matrices inhibited all evaluated PCR. As shown in this study, high-quality DNA was obtained from water, milk and apple juice samples using DNA extraction with the QIAamp DNA Mini Kit. DNA purification treatments removed PCR inhibitors before PCR amplification. This isolation and purification of DNA was used for evaluation of sensitivity of PCR methods for *C. parvum* oocysts detection. The DNA extraction method used in the

**Tab. 1.** Detection limit [oocysts per ml] of PCR for *Cryptosporidium parvum* in water, milk and apple juice.

| | DNA preparation | | | | | |
|---|---|---|---|---|---|---|
| | Heat lysis | | | DNA extraction | | |
| | Water | Milk | Apple juice | Water | Milk | Apple juice |
| Conventional PCR | $10^4$ | ND | ND | $10^3$ | $10^3$ | $10^3$ |
| Real-time PCR | $10^2$ | ND | ND | $10^2$ | $10^2$ | $10^2$ |
| Nested PCR | $10^2$ | ND | ND | $10^1$ | $10^1$ | $10^1$ |
| Modified nested PCR | – | – | – | $< 10^1$ | $< 10^1$ | $< 10^1$ |

ND - non-detectable, – - not performed.

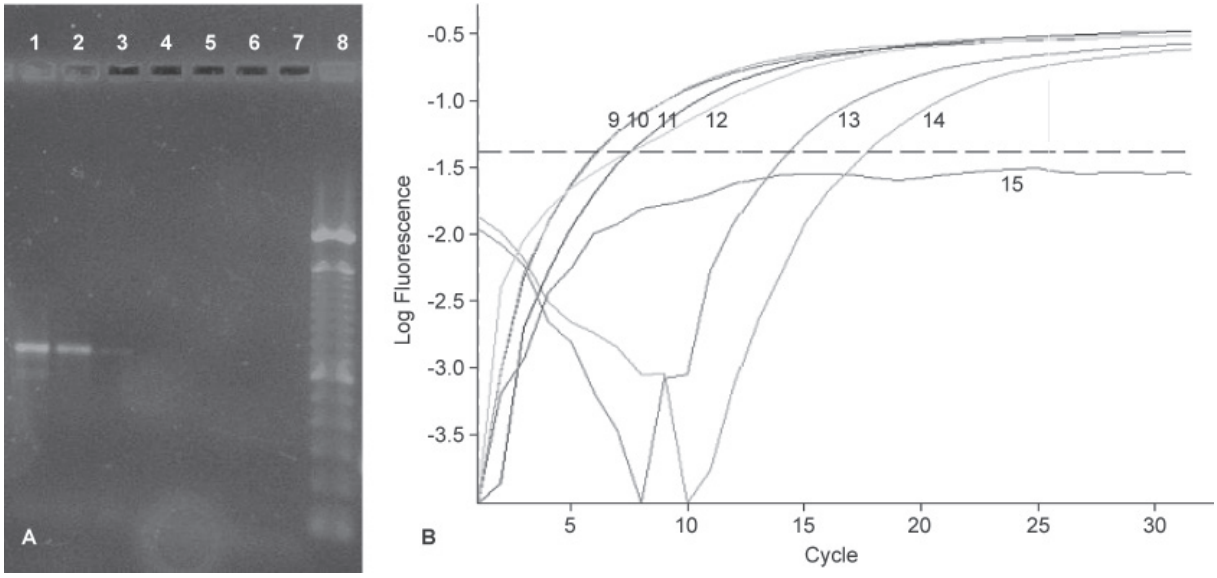


**Fig. 1A.** Electrophoresis of first round PCR amplification products from water contaminated with *C. parvum* oocysts.
1, 2, 3, 4, 5, 6 - extracted DNA *C. parvum* from oocysts suspension ranging from $10^5$ to $10^0$ oocysts per ml, amplification products 678 bp with primers CPrF1/R1; 7 - negative control; 8 - 100-bp DNA ladder.

**Fig. 1B.** A record of real-time PCR analysis.
9, 10, 11, 12, 13, 14 - DNA *C. parvum* after amplification of products 1–6, amplification products 118 bp with primers CPrF3/R3 and probe CPrP3-1; 15 - negative control.

PCR analysis of wastewater samples, where much more PCR inhibitors are normally present [10], gave better results.

With all evaluated PCR systems a maximal sensitivity of less than $10^1$ oocysts per ml in all evaluated matrices was achieved using the method developed by us (Fig. 1). The combination of primary round of conventional PCR with nested real-time PCR second round produced very good results of the PCR detection of *C. parvum* oocysts in all analysed matrices, reduced the detection time (positive samples detected in up to 20 cycles), laboratory work, operation with small-sized product in open system and handling of ethidium bromide. However, comparable detection limit of less than $10^1$ oocysts per ml was achieved with nested PCR

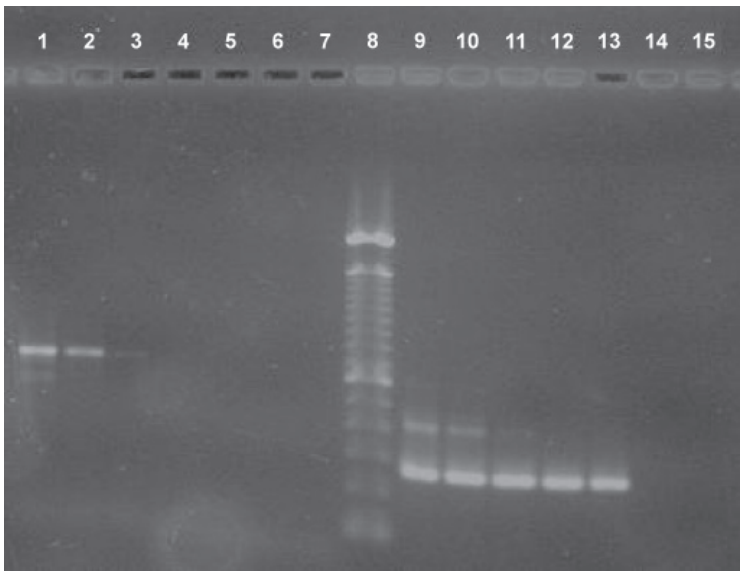


**Fig. 2.** Electrophoresis of nested PCR amplification products from decimal dilutions of water artificially contaminated with *C. parvum* oocysts.
1, 2, 3, 4, 5, 6 - extracted DNA *C. parvum* from oocysts suspension ranging from $10^5$ to $10^0$ oocysts per ml, amplification products 678 bp with primers CPrF1/R1; 7 - negative control; 8 - 100-bp DNA ladder; 9, 10, 11, 12, 13, 14 - DNA *C. parvum* after amplification of products 1–6, amplification products 285 bp with primers CPrF2/R2; 15 - negative control.

[9] (Fig. 2), although the elimination of the electrophoresis using ethidium bromide and operation with small-sized product of high concentration in open system presents an unquestionable advantage of our originally developed real-time PCR modification, especially for use in routine diagnostic laboratories.

Real-time PCR using a TaqMan probe is able to monitor the process in real time of the amplification reaction and may be applicable for quantification. There are only a few studies of rapid, specific and sensitive method available for quantifying the water-borne and food-borne parasite *Cryptosporidium parvum* [5, 6, 11]. Although a large number of PCR-based methods have been developed for the detection of *C. parvum* in water samples, they failed in food samples. Real-time PCR to detect oocysts *C. parvum* in water, milk and apple juice was performed with primer sets oriented on 18S rRNA. The detection limit of this method was $10^2$ oocysts per ml in all analysed matrices.

Finally, results of the study suggest that DNA isolation using chaotropic solid phase extraction and primary PCR in combination with nested real-time PCR represents an effective and sensitive tool for *Cryptosporidium parvum* detection in water, milk and apple juice samples.

## CONCLUSION

We showed that the improved nested PCR with real-time PCR second round developed in our laboratory was able to specifically detect *C. parvum* oocysts. Detection limit using extraction DNA with commercial kit used was less than $10^1$ oocysts per ml of water, milk and apple juice, respectively. Isolation of the DNA from oocysts in the presence of food matrices using heat lysis alone was shown to be an insufficient procedure, because the components from food matrices inhibited all evaluated PCR. Real-time PCR method is shown as a rapid, simple and user-friendly method for routine monitoring with satisfactory detection limit of $10^2$ oocyst per ml in water and food samples. Nested PCR is shown as the most sensitive method than real-time PCR method, but it is not suitable for routine work in diagnostic laboratory. On other side, modified nested PCR in combination with real-time PCR second round decreased the detection limit, shortened the time of analysis using closed-tube system.

**Acknowledgements**

This work was supported by Slovak Research and Development Agency in the project APVT 27-044402 "A study of the isolation and detection processes of the determination pathogen organisms *Cryptosporidium parvum* in a liquid phase".

## REFERENCES

1. Dawson, D.: Foodborne protozoan parasites. International Journal of Food Microbiology, *103*, 2005, pp. 207-227.
2. Di Pinto, A. - Tantillo, M. G.: Direct detection of *Cryptosporidium parvum* oocysts by immunomagnetic separation - polymerase chain reaction in raw milk. Journal of Food Protection, *65*, 2002, pp. 1345-1348.
3. Morgan, U. M. - Thompson, R. C. A.: PCR detection of *Cryptosporidium*: The way forward? Parasitology Today, *14*, 1998, pp. 241-244.
4. Laberge, I. - Ibrahim, A. - Barta, J. R. - Griffiths, M. W.: Detection of *Cryptosporidium parvum* in raw milk by PCR and oligonucleotide probe hybridization. Applied and Environmental Microbiology, *62*, 1996, pp. 3259-3264.
5. Fontaine, M. - Guillot, E.: An immunomagnetic separation - real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. Journal of Microbiological Methods, *54*, 2003, pp. 29-36.
6. Fontaine, M. - Guillot, E.: Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. FEMS Microbiology Letters, *214*, 2002, pp. 13-17.
7. Wu, Z. - Nagano, A. M. - Uga, S. - Kimata, I. - Iseki, M. - Takahashi, Y.: Specific PCR primers for *Cryptosporidium parvum* with extra high sensitivity. Molecular and Cellular Probes, *14*, 2000, pp. 33-39.
8. Abolmaaty, A. - El-Shemy, M. G. - Khallaf, M. F. - Levin, R. E.: Effect of lysing methods and their variables on the yield of *Escherichia coli* O157:H7 DNA and its PCR amplification. Journal of Microbiological Methods, *34*, 1998, pp. 133-141.
9. Bialek, R. - Binder, N. - Dietz, K. - Joachim, A. - Knobloch, J. - Zelck, U. E.: Comparison of fluorescence, antigen and PCR assays to detect *Cryptosporidium parvum* in fecal specimens. Diagnostic Microbiology and Infectious Disease, *43*, 2002, pp. 283-288.
10. Jiang, J. - Alderisio, K. A. - Singh, A. - Xiao, L.: Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. Applied and Environmental Microbiology, *71*, 2005, pp. 1135-1141.
11. Guy, R. A. - Payment, P. - Krull, U. J. - Horgen, P. A.: Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Applied and Environmental Microbiology, *69*, 2003, pp. 5178-5185.

Received 9 January 2007; revised 1 February 2007; accepted 1 February 2007.