Journal of Industrial Microbiology & Biotechnology (1998) 21, 150–166

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PCR detection of *Cryptosporidium parvum* in environmental samples—a review of published protocols and current developments

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Since 1991 more than 30 PCR protocols have been published, which show a potential to replace the current microscopic detection method for *Cryptosporidium parvum* in environmental samples and food. This review provides a synoptic comparison of these protocols with respect to the following features: isolation and purification of oocysts from tested matrices, elimination of free DNA, viability and infectivity assessment, release of nucleic acids, nucleic acid extraction, type of PCR (PCR, RT-PCR, internal-standard-PCR, *in situ* PCR, TaqMan-PCR), primary product detection, additional specificity control, secondary product detection, reported sensitivity, cross-reaction with other *Cryptosporidium* species, and target and sequence information such as amplicon length, primer sequences, multiple copy target, presence of strain-specific differences in the amplicon, GenBank accession numbers and gene function. The results demonstrate that problems like PCR inhibition, viability assessment, and the requirement of an extreme sensitivity have been solved. PCR assays would be most valuable to control presence-absence standards in defined matrix volumes, and the setup of such standards would very much contribute to a rapid introduction of this awaited technology into routine monitoring of environmental, water and food samples, and to a further standardization of the various protocols. It can be expected that satisfactory solutions for quantification will be found for a growing number of PCR-based assays. Systematic field evaluation and interlaboratory studies will complement our present knowledge of these methods in the near future.

Keywords: *Cryptosporidium parvum*; detection; PCR; environmental samples; water; food

Introduction

Cryptosporidium parvum is a protozoan parasite that can infect a wide variety of vertebrates. It is frequently observed in young cattle and sheep. In immunocompetent persons and in young animals it can cause a severe but self-limiting diarrhea and various other gastro-intestinal symptoms. It is transmitted by the fecal-oral route, and human infections and epidemics have been linked to the consumption of fecally contaminated drinking water, swimming pool water, recreational water, raw and insufficiently pasteurized milk, cider and berries [13]. *Cryptosporidium* oocysts have been experimentally accumulated in oysters [19].

The complete life cycle of *Cryptosporidium parvum*, including sexual and asexual multiplication, takes place in a single host. The infection starts with the oral uptake of infective sporulated oocysts. Inside each oocyst there are four sporozoites, which actively penetrate the oocyst wall when the oocysts arrive in the small intestines. Factors which trigger this excystation are temperature, changes in pH and the presence of bile salts and pancreatic enzymes. After excystation the sporozoites infect mucosal cells, they multiply and in the end of the intestinal life cycle, large amounts of sporulated oocysts are shed with the stools. Shed oocysts can immediately infect other hosts but can also remain viable for several months in cool and humid environments.

C. parvum is assumed to be the only species which causes disease in humans. Seven other species, which are considered to be valid, have been identified in animals: *C. muris* in mammals, *C. wrairi* in guinea pigs, *C. felis* in domestic cats, *C. meleagridis* and *C. baileyi* in birds, *C. nasorum* in fish and *C. serpentis* in reptiles.

Due to the low infectious dose of *C. parvum*, extremely sensitive detection methods are required for water and food analysis. The conventional direct immunofluorescence methods that are currently in use for microscopic detection of the parasites in water samples are very laborious. The diagnosis of the antibody-labeled oocysts depends very much on personal experience. The reproducibility within and between laboratories is often not very good and, unless additional vital dyes are used, the simple staining of the oocyst wall does not give any information about the viability and infectivity of the detected organism. The available antibodies are not species-specific and crossreact at least with one of the other known *Cryptosporidium* species. In addition, there is the possibility of confusing the target organisms with autofluorescing or crossreacting algal cells and other detritus in the concentrates that have to be examined. To overcome these difficulties, enormous efforts have been made in recent years to develop sensitive and specific molecular detection methods for this parasite. One of them is the polymerase chain reaction.

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Received 5 May 1998; accepted 7 September 1998

Overview of PCR-based methods applicable for *Cryptosporidium* detection

Source of test organisms

When *Cryptosporidium* oocysts are required for experimental purposes, eg, to test or establish a PCR-based detection system, they have to be isolated from naturally infected persons or animals, or they have to be propagated in test animals, usually in neonatal calves or rodents. At the present time no reference organisms are available from national culture collections such as ATCC or DSM, but there are some commercial organizations where live or inactivated *Cryptosporidium parvum* oocysts can be obtained.

PCR inhibition

PCR can be inhibited by a variety of substances, which can be present not only in stool samples but also in environmental materials and foods. Johnson *et al* [25] found a 100–1000 fold reduction in sensitivity in some water samples compared to pure oocyst preparations. This inhibition was reduced when flow cytometry was used before PCR and by inclusion of the chelating agent Chelex100 (BioRad, CA, USA) in the sample during freeze-thaw extraction. Sluter *et al* [47] tried to characterize inhibitors for *Cryptosporidium* PCR in raw lake water by gel filtration experiments. They found that the most important inhibitors are not part of the particulate fraction of this matrix, but part of the soluble fraction with a molecular size of less than 27 kDa. This is about the size range of humic acids. In order to achieve the required sensitivity of only a few or even one oocyst, it is therefore essential to separate the oocysts from the sample matrix and make the oocyst preparations as pure as possible. The most frequently applied separation method is sucrose flotation or Percoll density gradient centrifugation. A more sophisticated approach is the use of fluorescence activated cell sorting (FACS) and immunomagnetic separation (IMS). Further purification can be achieved by DNA or RNA extraction methods as described later in the text. Wiedenmann *et al* [54] have reported a *ca* 1000-fold increase in sensitivity, after a silica extraction was introduced into the PCR protocol of Filkorn *et al* [20], though later results of Deng *et al* [18] suggest that immunomagnetic capture of oocysts alone is apparently sufficient to remove the majority of PCR inhibitors.

Assessment of viability

PCR detects nucleic acids. It cannot selectively detect viable organisms. It also detects nucleic acids of dead organisms as has been shown not only for *Cryptosporidium* oocysts but also for various bacteria and viruses in disinfected drinking water. Basically two different ways to solve this problem have been described for *Cryptosporidium* PCR: the combination of PCR with *in vitro* excystation, and the detection of mRNA by RT-PCR.

In 1994 Filkorn *et al* [20] published a series of experiments, in which they found that: (1) PCR was positive when free DNA from dead and disintegrated *Cryptosporidium* oocysts was present in a sample material; (2) PCR was negative when this free DNA was destroyed by a DNA digest, though the reaction mix still contained viable oocysts; (3) PCR was positive again when an *in vitro* excystation assay was performed after the DNA digest; and (4) PCR was negative when an *in vitro* excystation was performed after a DNA digest, but with oocysts that had been killed by heating. The authors concluded that the PCR protocol itself does not destroy intact oocyst walls and no signal can be achieved when oocysts are merely added to the PCR mix. But when free DNA, which might be present in a sample, is destroyed or removed and oocysts are consequently submitted to an *in vitro* excystation protocol, PCR can be performed selectively with the nucleic acids from excysted sporozoites which have, by their active excystation, proven to be viable. A positive PCR reaction can then be interpreted as the presence of viable oocysts in the sample [20]. This method becomes feasible, because excysted sporozoites are much more fragile than oocysts, and can be lysed by procedures which leave dead oocysts intact. Complete protocols which combine *in vitro* excystation assays and PCR for viability assessment have also been published by Wagner-Wiening *et al*, Wiedenmann *et al* and Deng *et al* [50,54,18]. The main differences between these assays are the following: Wagner-Wiening *et al* performed the DNase digest *after in vitro* excystation, and reported the sensitivity on the basis of numbers of purified sporozoites not oocysts. This makes it difficult to compare the sensitivity of this assay to others. As already mentioned, sporozoites are relatively fragile, and one cannot assume a strict mathematical correlation between the number of sporozoites and the number of oocysts, eg, in a way that eight sporozoites are equivalent to two oocysts, when the overall assay sensitivity *including in vitro* excystation has to be evaluated. Deng *et al* basically reproduced the method described by Filkorn *et al*. They did, however, *not* perform a DNase digest, but have isolated the oocysts by *immunomagnetic capture*. They have reported no false positive results from free DNA in oocyst suspensions which were inactivated by heating or by treatment with 10% formalin or 10% ammonia. It may therefore be concluded that immunomagnetic capture of oocysts represents an alternative method to exclude false positive results due to free DNA. The protocol of Wiedenmann *et al* is a development of the Filkorn method with largely improved sensitivity but without fundamental changes in the way viability is assessed.

A viability assessment by detection of mRNA has the advantage that it does not depend on a preceding biological process like *in vitro* excystation. The removal of DNA, which would produce false positive results, can be done either by a DNA digest or by an mRNA recovery method, which is selective for mRNA, eg oligo(dT)-linked magnetic beads [49] or oligo(dT)-linked cellulose [43].

The mRNA method is based on two additional preconditions. The first one is that the half-life of the mRNA target is very short, and that mRNA cannot be recovered in detectable amounts from dead organisms. This precondition is not met by all kinds of mRNA. While some kinds of mRNA are degraded within several minutes inside the organism, others remain intact for up to several days [27]. As the work of Mahbubani *et al* has shown, eg, the mRNA of the giardin protein of *Giardia* cysts can be recovered under experimental conditions from cysts killed by heating or monochloramine exposure [33]. For this reason, the mRNA of heat shock proteins (hsps) is supposed to be a

more suitable target. The mRNA of *Drosophila* hsps has been demonstrated to be unstable at ambient temperatures [17] and it may be assumed that hsp-mRNA is not present in dead oocysts from environmental samples. This assumption has, however, not yet been experimentally verified. The second precondition is that the mRNA target is always present in viable organisms. As heat shock proteins play an important role for the correct folding of polypeptides during their synthesis in the cell, they are normally present at a low level even without induction by heat or other stimuli [27]. Stinear *et al* performed a heat treatment of 20 min at 45°C on their *Cryptosporidium* samples in order to induce the production of mRNA from heat shock protein 70 (*hsp70*), which then served as the target for an RT-PCR [49]. The heat treatment, however, was not a necessary precondition for a positive RT-PCR result, and the authors conclude that other stress factors, which occur during sample preparation, are also capable of inducing *hsp70*-mRNA production. For *Escherichia coli*, a temperature increase from 30°C to 42°C induces an increase of the concentration of hsp70, which reaches a maximum after *ca* 5 min. After 10 min the concentration of hsp70 has already dropped again to a level which is only *ca* 2–4 times as high as before the temperature increase [27]. This may be an additional explanation, why Stinear *et al* could not find any dramatic change in their RT-PCR results after a heat treatment of 20 min.

While some general questions concerning the viability assessment by mRNA detection remain to be evaluated, there is more experimental experience with the *in vitro* excystation assay. It has been used to evaluate viability after storage of oocysts under different environmental pressures and after disinfection procedures [8,9,21,41]. Compared to neonatal mouse infectivity, *in vitro* excystation seems to rather overestimate than underestimate infectivity, when oocysts are treated with ozone or other common water disinfectants [21]. However, for a detection method which is applied to drinking water samples or food, this is certainly more acceptable than if it were the other way round. In addition, the discrepancy between *in vitro* excystation and neonatal mouse infectivity might also be due to an experimental limitation of the *in vitro* excystation assay, as it would be necessary to count microscopically thousands of oocysts to demonstrate a reduction of more than three log units.

When a viability assay is to be performed on *Cryptosporidium* samples, the sample material should not be preserved by addition of chemicals which can reduce viability, such as formalin or potassium dichromate [7].

Assessment of infectivity

Rochelle *et al* [44] especially stress that there is a difference between viability and infectivity, and state that for an accurate assessment of the risk posed to public health, water works need to know whether oocysts which are present in a water sample are infective, not only viable. As it is impractical to perform animal infectivity assays on a routine basis, cell culture infectivity is proposed as an equivalent. Rochelle *et al* [44,45] report that mRNA of the *hsp70* gene of *C. parvum* could be detected in a CaCo-2 cell culture within 2 h after inoculation with 1000 oocysts. However, after inoculation with only one oocyst, an incubation time of 48 h was necessary. As an alternative method for detecting infectious foci in a cell culture, *in situ* PCR has been used [45]. But *in situ* PCR results were only preliminary and no incubation time has been specified for this assay.

This means that from the present state of our knowledge, testing for cell culture infectivity provides an additional criterion for interpretation of the presence of *Cryptosporidium* oocysts in food samples, and it may provide an increase in sensitivity. On the other hand, this additional information requires a longer analysis time (2 days more for low numbers of *Cryptosporidium* oocysts) and a far more complex methodology, which needs additional laboratory equipment and staff experience, if cell culture techniques are not yet established. Cell culture infectivity assays do not only depend on *in vitro* excystation as a first step, but on an additional biological process: the invasion of excysted sporozoites into vital cells and a consequent multiplication. This can also be looked upon as an additional source of error, and it must be discussed how food suppliers, water works or health authorities should react, if viable organisms can be detected, but a cell culture infectivity assay is negative.

Release of nucleic acids

Cryptosporidium oocysts have very robust walls, and, as mentioned, the temperature changes during PCR are not capable of releasing the nucleic acids from intact oocysts, and even boiling is insufficient. Basically there are two different ways of coping with this problem. One is to apply rather vigorous methods, which destroy or disrupt oocysts and internal sporozoites regardless of their viability. These are: proteinase K digestion for up to 48 h, several freeze-thaw cycles (usually in liquid nitrogen and hot water), or sonication. Sluter *et al* [47] compared the three methods and found that proteinase K digestion did not give superior results compared to three cycles of freezing and thawing in solid CO_2-ethanol and a 37°C waterbath. Sonication (three times 5 min in a waterbath) was equivalent to the freeze-thaw procedure, but was regarded as less practical, and certainly depends on the kind of sonicator used. After electroporation (two pulses of 2.5 kV, 129 Ω), the PCR signal was fainter than after freeze-thaw. In these authors' experience, five cycles of freezing and thawing did not improve PCR detection over the results obtained by three cycles. This is in apparent contrast to the results of Laberge *et al* [29], who recommend 10 cycles instead of five (liquid nitrogen/65°C) and an additional proteinase K treatment in order to increase the DNA yield. In any case, it seems to be necessary to perform freeze-thaw procedures in the presence of detergents, as according to our own experience even 10 cycles of freezing in CO_2-ethanol and thawing at 70°C are not sufficient to reliably disrupt *Cryptosporidium* oocysts in watery suspensions.

The second method to make the nucleic acids available for PCR detection is to perform an *in vitro* excystation, as described above, and to release the nucleic acids from excysted sporozoites. To release nucleic acids from sporozoites is far more easy. It can be done simply by boiling [18,20] or by other rapid procedures like short proteinase

Table 1 Synopsis of *Cryptosporidium* detection assays: isolation and purification of oocysts from tested matrix, elimination of free DNA, viability assessment, release of nucleic acids, nucleic acid extraction

Authors	Isolation and purification of oocysts from tested matrix	Elimination of free DNA	Assessment of viability or cell culture infectivity	Release of nucleic acids	Nucleic acid extraction
Laxer (1991) [30]	sucrose gradient centrifugation and bleach-sterilization (5.25% NaHOCl 10 min, 0°C)	no	no	TE-SDS-proteinase K digestion (48 h)	phenol and chloroform extraction, ethanol precipitation, dialysis against TE-buffer
Cai (1992) [6]	ether lipid extraction and NaCl-gradient centrifugation	no	no	freeze-thaw treatment	not specified
Johnson (1993) [25]	not specified	no	no	freeze-thaw treatment	no
Ranucci (1993) [40]	not specified	no	not specified	TE-SDS-proteinase K digestion (3 h) of excysted oocysts	phenol extraction, ethanol precipitation
Webster (1993) [52]	sieving (53 μm) and salt flotation	no	no	3× freeze-thaw in TE-dithiothreitol buffer, heating (20 min, 90°C)	no
Awad-el-Kariem (1994) [2]	ether lipid extraction and NaCl-flotation	no	no	TE-SDS-proteinase K digestion (48 h)	phenol and chloroform extraction, ethanol precipitation
Filkorn (1994) [20]	sucrose flotation	DNase I-digestion before *in vitro* excystation	*in vitro* excystation: gall acid (4 h, 37°C)	DNA release from sporozoites by boiling (15 min)	no
Carraway (1994) [10]	sucrose gradient centrifugation and bleach-sterilization (5.25% NaHOCl 10 min, 0°C)	no	no	freeze and thaw treatment; SDS-proteinase K digestion (overnight 37°C)	phenol/chloroform extraction; ethanol precipitation
Johnson (1995) [26]	sucrose/Percoll gradient centrifugation or IMS or flow-cytometry	no	no	6× freeze-thaw in 20% Chelex 100	no
Wagner-Wiening (1995) [50]	ether-extraction and Percoll gradient centrifugation	DNase digest after *in vitro* excystation	*in vitro* excystation: trypsin, gall acid (1 h, 37°C)	DNA release from sporozoites by TE-SDS-proteinase K-dithiothreitol digestion (1 h, 58°C)	phenol-chloroform-isoamyl alcohol extraction; isopropanol-glycogen precipitation, washing with isopropanol and drying
Wiedenmann (1996) [54]	sieving and sucrose flotation	DNase I digest before *in vitro* excystation	*in vitro* excystation: aHBSS (30 min, 37°C), trypsin, gall acid (⩾30 min, 37°C)	DNA release from sporozoites by incubation in TE-guanidinium thiocyanate-Triton X-100 buffer (10 min at room temperature)	extraction with diatom particles, washing with TE-guanidinium thiocyanate buffer, ethanol and acetone
Leng (1996) [31]	sucrose gradient centrifugation	no	no	4× freeze-thaw and heating (3 h, 75°C) in Nonidet P 40/SDS/NaOH/ proteinase K lysis-buffer	PCR Select-III spin columns (5′Prime-3′Prime, Boulder, CO)
Leng (1996) [32]	sucrose gradient centrifugation	no	no	4× freeze-thaw and heating (3 h, 75°C) in Nonidet P 40/SDS/NaOH/ proteinase K lysis-buffer	PCR Select-III spin columns (5′Prime-3′Prime, Boulder, CO)

Continued

Table 1 Continued

Authors	Isolation and purification of oocysts from tested matrix	Elimination of free DNA	Assessment of viability or cell culture infectivity	Release of nucleic acids	Nucleic acid extraction
Mayer (1996) [34]	Percoll-sucrose gradient centrifugation	no	no	EDTA-proteinase K digestion (2 h, 65°C), 6× freeze and thaw in TE-SDS-proteinase K buffer	phenol-chloroform-extraction; chloroform-extraction isopropanol precipitation, purification by Centricon-100 (Amicon, Beverley, MA) concentrator and Sephadex 200 column (Pharmacia, Piscataway, NJ)
Webster (1996) [53]	IMS	no	no	5× freeze-thaw treatment in TE-dithiothreitol-buffer	no
Laberge (1996) [29]	Stool samples: sucrose gradient centrifugation Milk: incubation with Bacto-Trypsin and Triton X-100 (30 min, 50°C) and centrifugation	no	no	10× freeze-thaw treatment in TE-sarcosyl-proteinase K-buffer, digestion with additional proteinase K (1 h, 55°C)	Isogene kit (Perkin-Elmer Cetus, Norwalk, CT)
Stinear (1996) [49]	not evaluated	no	by detection of hsp70 mRNA after heat induction (20 min, 45°C)	5× freeze-thaw treatment in TE-LDS-dithiothreitol-buffer	mRNA-extraction with oligo $(dT)_{25}$-linked magnetic beads
Balatbat (1996) [3]	1:4 dilution in TES buffer, centrifugation ($2000 \times g$), centrifugation of the supernatant ($7000 \times g$) and bleach-sterilisation (5.25% NaHOCl)	no	no	TES-proteinase K digestion (2 h, 60°C)	phenol-chloroform-isoamyl alcohol extraction; ethanol precipitation
Rochelle (1996) [43]	sucrose gradient and decontamination by incubation with 1.2% NaHOCl (10 min, 0°C)	no	a) *in vitro* excystation; PBS (1 h, 37°C) trypsin, gall acid (2 h, 37°C), filtration of sporozoites through 2-μm filter, b) cell culture infection, 2–48 h incubation, c) detection of mRNA	RNA and DNA from infected cells with guanidine, phenol, chloroform	oligo (dT) cellulose kit (Sigma)
Bonnin (1996) [5]	PBS-ether centrifugation and discontinuous Percoll density gradient or salt flotation [4]	no	no	15× freeze-thaw treatment in Tris-Tween 20 buffer, boiling (15 min, 100°C), centrifugation	no
Morgan (1996) [37]	filtration of fecal samples through gauze, PBS-ether sedimentation, Ficoll-density centrifugation and incubation in 10% NaOHCl (10 min, 0°C) or dilution of the fecal samples with PBS and TE (1:160)	no	no	3× freeze-thaw treatment in TE-buffer, boiling (5 min), centrifugation	no

Continued

Table 1 Continued

Authors	Isolation and purification of oocysts from tested matrix	Elimination of free DNA	Assessment of viability or cell culture infectivity	Release of nucleic acids	Nucleic acid extraction
Gobet (1997) [22]	mixing of fecal samples with 0.35% NaHOCl, sieving, ether-extraction, NaCl gradient centrifugation, filtration through 3 μm filter, rinsing of filter and centrifugation	no	no	15× freeze-thaw treatment in Tris-Tween 20 buffer, boiling (15 min, 100°C), centrifugation	no
Rochelle (1997) [46]	'Purified preparations': treatment not specified	no	no	freeze-thaw treatment	no
	'Unpurified concentrates': no treatment			SDS-proteinase K-buffer (30 min, 37°C), addition of CTAB (15 min, 65°C),	Phenol-chloroform-isoamylalcohol-extraction, isopropanol precipitation, washing in ethanol, vacuum desiccation
	Seeded environmental water concentrates: no further purification			Incubation in TE-sarcosyl-proteinase K-buffer (1 h, 37°C), incubation with additional NaCl and CTAB (30 min, 65°C), 1× freeze and thaw	Phenol-chloroform, extraction, isopropanol precipitation, washing in ethanol, vacuum desiccation
Patel (1997) [39]	salt flotation	no	no	repeated cycles of freeze-thaw treatment	no
Rochelle (1997) [44]	Stool samples: sucrose gradient and decontamination by incubation with 0.5% NaHOCl in PBS (10 min, 0°C) Environmental samples: sucrose flotation	no	Detection of mRNA or DNA after *in vitro* excystation and cell culture-infection: 1.1% NaHOCl (10 min, 0°C) PBS (1 h, 37°C) trypsin, gall acid (2 h, 37°C), filtration of sporozoites through 2-μm filter, infection of cell culture, 2–48 h incubation	mRNA (from cell culture): cell lysis with SDS (5 min, 20°C) DNA (from environmental samples and pure cultures): incubation in TE-sarcosyl-proteinase K-buffer (1 h, 37°C), incubation with additional NaCl and CTAB (30 min, 65°C) total RNA and DNA (from cell culture): homogenization of cells with TriReagent kit®	mRNA: from cell lysate with oligo (dT) cellulose kit DNA: phenol-chloroform-extraction, isopropanol precipitation, washing in ethanol, vacuum desiccation, resuspending in water, spin column purification total RNA and DNA (from cell culture): TriReagent kit® (Molecular Research Center, Cincannati, OH)
Spano (1997) [48]	formol-ether-extraction	no	no	4× freeze and thaw treatment in 0.1% SDS, 10 min boiling Incubation in TE-guanidinium thiocyanate-Triton X-100 buffer (30 min, 20–25°C)	Phenol-chloroform extraction, ethanol precipitation Extraction with silica- particles, washing with TE-guanidinium thiocyanate buffer, ethanol and acetone

Continued

Table 1 Continued

Authors	Isolation and purification of oocysts from tested matrix	Elimination of free DNA	Assessment of viability or cell culture infectivity	Release of nucleic acids	Nucleic acid extraction
Deng (1997) [18]	Immunomagnetic capture	no	*in vitro* excystation: bovine bile and $NaHCO_3$ (4 h, 37°C)	incubation of washed sporozoites for 10 min at 90°C and for 5 min at 4°C	no
Carraway (1997) [12]	not specified	no	no	not specified	not specified
Wagner-Wiening (1997) [51]	not specified	no	no	permeabilization of oocyst wall	not necessary
Rochelle (1997) [45]	concentration method not specified, decontamination of water-concentrates by incubation with 0.5% NaHOCl in PBS (30 min, 4°C)	no	*in vitro* excystation plus cell culture infection (2–72 h incubation), *in situ* PCR in infected cells (only general information; no exact protocols specified)	not necessary	not necessary
Chung (1997) [16]	not specified	no	no	lysis in TE-sarcosyl-proteinase K-buffer, 10× freeze and thaw additional Proteinase K (30 min, 37°C)	Qiagen columns
Sluter (1997) [47]	discontinuous sucrose gradient or isopycnic Percoll gradient	no	no	3× freeze and thaw	phenol-chloroform extraction, ethanol precipitation
Krüger (1998) [28]	Immunomagnetic capture or sucrose flotation	DNase I digest before *in vitro* excystation	*in vitro* excystation: aHBSS (30 min, 37°C), trypsin, gall acid (⩾1 h, 37°C)	DNA release from sporozoites by incubation in TE-guanidinium thiocyanate-Triton X-100 buffer (10 min at room temperature)	extraction with diatom particles, washing with TE-guanidinium thiocyanate buffer, ethanol and acetone

K treatment [50] or application of guanidinium-iso-thiocyanate in the presence of detergents [43,54]. A disadvantage of this method is that it requires an additional biological process which depends on the physiological condition of the organism and is to some extent variable. The excystation time, eg, can vary between a few minutes and 4 h [42]. When nucleic acids are released from excysted sporozoites only, PCR results are indicative for viable organisms, as already explained. Therefore, *in vitro* excystation should only be used when viable organisms have to be detected, and the presence of dead organisms in the sample material would be unimportant.

Nucleic acid extraction

The volumes which can be analyzed by PCR are usually smaller than 80 μl. To reduce a sample to such a small volume, and to remove PCR inhibitors, many authors apply extraction methods for nucleic acids like phenol-chloroform extraction and ethanol precipitation, silica extraction, isolation with magnetic beads or commercially available extraction kits. Apparently, nucleic acid extraction procedures are not absolutely necessary. Some authors have found, that if oocyst preparations are sufficiently clean, sensitive detection of the target organisms is possible even without additional DNA or RNA extraction [5,18,22,25,26, 37,46,52,53]. These authors have achieved the required purity by thorough washing of the oocyst suspensions or sporozoites and/or immunomagnetic separation.

Target sequence

The choice of the PCR target sequence in the genome of the organism of interest is basically arbitrary. In a diagnostic PCR it is not even necessary that the function of a target gene sequence is well known. The target sequence can, however, influence the result with respect to sensitivity and specificity.

The number of *C. parvum* sequences which are accessible via databases like GenBank or EMBL has been small for years but is now dramatically increasing. So far, 932 *C. parvum* sequences are listed in GenBank. The protocols in the 33 reviewed papers, however, refer to only 10 different gene sequences. Three of them are DNA sequences which code for 18S rRNA (X64340-3, L16996, S76662), three code for oocyst wall proteins (U35027, Z22537, M95743), one codes for a surface antigen (U83169), one codes for heat shock protein 70 (U11761), and two sequences are of unknown function (M59419, L01269). Compared to *C. parvum*, other *Cryptosporidium* species are represented in GenBank to a much smaller extent (four *C. muris* sequences, three *C. wrairi* sequences and one *C. bai-*

Table 2 Synopsis of *Cryptosporidium* detection assays: type of PCR, primary product detection, additional specificity control, secondary product detection

Authors	Type of PCR	Primary product detection	Additional specificity control	Secondary product detection
Laxer (1991) [30]	PCR	PAGE, agarose	restriction digestion, southern blot and oligonucleotide hybridization	chemiluminescence
Cai (1992) [6]	PCR	not specified	no	no
Johnson (1993) [25]	PCR	agarose	restriction digestion	agarose
Ranucci (1993) [40]	PCR	agarose	no	no
Webster (1993) [52]	PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Awad-el-Kariem (1994) [2]	PCR	agarose	nested PCR and restriction digestion	agarose
Filkorn (1994) [20]	PCR	agarose	no	no
Carraway (1994) [10]	PCR	agarose	no	no
Johnson (1995) [26]	PCR	agarose	dot blot and oligonucleotide hybridization	chemiluminescence
Wagner-Wiening (1995) [50]	PCR	agarose	nested PCR restriction digestion	agarose
Wiedenmann (1996) [54]	PCR	agarose	no	no
Leng (1996) [31]	PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Leng (1996) [32]	PCR	agarose	RFLP	agarose
Mayer (1996) [34]	PCR	agarose	southern blot and oligonucleotide hybridization nested PCR (283 bp)	chemiluminescence agarose
Webster (1996) [53]	PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Laberge (1996) [29]	PCR	agarose	slot blot and oligonucleotide hybridization	chemiluminescence
Stinear (1996) [49]	RT-PCR	PAGE, agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Balatbat (1996) [3]	PCR	agarose	nested PCR (194 bp) southern blot and oligonucleotide hybridization	agarose chemiluminescence
Rochelle (1996) [43]	RT-PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Bonnin (1996) [5]	PCR	agarose	RFLP-analysis	agarose
Morgan (1996) [37]	PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Gobet (1997) [22]	PCR	agarose	no	no
Rochelle (1997) [46]	PCR	agarose	southern blot and oligonucleotide hybridization	chemiluminescence
Patel (1997) [39]	PCR	agarose	restriction digestion	agarose
Rochelle (1997) [44]	RT-PCR PCR	agarose	southern blot and oligonucleotide hybridization, slot blot and oligonucleotide hybridization	chemiluminescence
Spano (1997) [48]	PCR	agarose	restriction digestion	agarose
Deng (1997) [18]	PCR	agarose	nested PCR (210 bp) restriction digestion southern blot and oligonucleotide hybridization	agarose agarose chemiluminescence
Carraway (1997) [12]	PCR	agarose	nested PCR of the 2262 bp-amplicon (781 bp) restriction digestion	agarose
Wagner-Wiening (1997) [51]	*in situ* PCR	epifluorescence-microscopy	*in situ* oligonucleotide hybridization	no
Rochelle (1997) [45]	*in situ* PCR *in situ* RT-PCR	epifluorescence-microscopy	*in situ* oligonucleotide hybridization	no
Chung (1997) [16]	IS-PCR	agarose ELISA[a]	nested PCR not specified	not specified
Sluter (1997) [47]	PCR	agarose	no	no
Krüger (1998) [28]	TaqMan-PCR	luminescence-spectrometer	oligonucleotide probe hybridization	no

IS = Internal Standard.
[a]Digene Sharp Signal system (colorimetric ELISA after biotin-streptavidin capture of DNA-RNA hybrids).

leyi sequence). At present it is therefore impossible to check primers by computer analysis for possible cross-reactions with all other *Cryptosporidium* species. If such cross-reactions have to be excluded, it is necessary to test the primers experimentally with the other species. These, however, are not readily available, and none of the described primer pairs has been tested with all of them. Several authors have evaluated the specificity of their primers with *C. baileyi* and *C. muris* [2,14,25,26,30,32,43,44,46,49,52,53]. Only three protocols have been tested with *C. wrairi* [15,26,48], and one protocol was checked against *C. serpentis* [37]. Champliaud *et al* [14] determined the specificity of primers of eight different assays [2,5,26,29,30,37,44,50] with respect to *C. muris*, *C. baileyi* and *C. meleagridis*. *C. parvum* and *C. meleagridis* could not be differentiated even after restriction enzyme digestion obtained from three of the target genes. The primer and probe system, which has been most extensively applied and has been evaluated by independent authors, is the one which was first described by Laxer *et al* in 1991 [3,14,15,18,20,22,29,30,31,46]. The primers give negative results for *C. muris* [14,18,29,46] and *C. baileyi* [14,22,46]. They give positive results for *C. meleagridis*, as do all other seven primer systems which have been evaluated with this latter species [14]. Chrisp *et al* reanalyzed the amplicon which is generated by the Laxer primers and have checked the primer specificity also against *C.*

Table 3 Synopsis of *Cryptosporidium* detection assays: tested matrices, reported sensitivity, crossreaction with other *Cryptosporidium* species

Authors	Tested matrices	Reported sensitivity	Crossreaction with other *C.* species
Laxer (1991) [30]	purified oocysts	30 fg *C. parvum* DNA (1.PCR)	negative for *C. muris* [14,18,29,46] and *C. baileyi* [14,22,46], negative for *C. wrairi* only after application of internal probe [15] (prl, see Table 5), positive for *C. meleagridis* [14]
Cai (1992) [6]	purified oocysts	not determined	not determined
Johnson (1993) [25]	a) purified oocysts b) environmental samples (seeded)	a) 10–100 oocysts (1.PCR) b) 500–10 000 oocysts (1.PCR), depending on kind of sample	PCR: *C. baileyi* and *C. muris* positive. Restriction digestion: different profiles for *C. parvum*, *C. baileyi* and *C. muris*
Ranucci (1993) [40]	purified oocysts	40 oocysts (1.PCR)	not determined
Webster (1993) [52]	purified oocysts	2000 oocysts (1.PCR) 20 oocysts (southern blot and oligonucleotide hybridization)	PCR: *C. baileyi* pos. *C. muris* neg. oligonucleotide hybridization: *C. baileyi* neg.
Awad-el-Kariem (1994) [2]	purified oocysts	not determined	PCR and nested PCR: *C. baileyi* and *C. muris* pos. [2,14] *C. meleagridis* pos. [14] restriction digestion: different profile for *C. parvum*, *baileyi* and *muris* [2] same profile for *C. parvum* and *meleagridis* [14]
Filkorn (1994) [20]	purified oocysts	not determined	see Laxer
Carraway (1994) [10]	purified oocysts	not determined	not determined
Johnson (1995) [26]	a) purified oocysts	a) 47–900 oocysts (1.PCR), 0.9–9 oocysts (dot blot), depending on source and age of sample.	*C. meleagridis, muris, baileyi*, positive [14,26] *C. wrairi* positive [26]
	b) environmental samples	b) 100–1000-fold reduced sensitivity in comparison to (a)	
	c) environmental samples after IMS	c) 125 oocysts (dot blot), lower numbers not evaluated	
	d) environmental samples after flow cytometry	d) 58 oocysts (dot blot)	
Wagner-Wiening (1995) [50]	purified oocysts	30 sporozoites (1.PCR), sometimes 10 sporozoites (nested PCR)	not determined *C. muris* and *C. baileyi* negative *C. meleagridis* positive [14]
Wiedenmann (1996) [54]	purified oocysts	10–20 oocysts (1.PCR)	see Laxer
Leng (1996) [31]	a) purified oocysts	a) 100 oocysts (1.PCR), 0,1 oocyst (southern blot and oligonucleotide hybridization)	see Laxer
	b) diluted stool samples	b) 10 000 oocysts ml^{-1} by PCR and 100 oocysts ml^{-1} after oligo hybridization = 2000 oocysts (1.PCR); 2 oocyst (southern blot and oligonucleotide hybridization)	
Leng (1996) [32]	purified oocysts	not determined	differentiation of *C. parvum, C. baileyi* and *C. muris* by RFLP
Mayer (1996) [34]	environmental water samples	370 oocysts $liter^{-1}$ (nested PCR), exact number of oocysts per PCR is not calculable from the reported data	not determined
Webster (1996) [53]	fecal samples: a) crude b) purified by IMS	a) 100 oocysts ml^{-1} fecal sample = 0.1 oocyst/PCR (southern blot and oligonucleotide hybridization) b) 5 oocysts ml^{-1} fecal sample = 0.8 sporozoites–2 oocysts/PCR) (southern blot and oligonucleotide hybridization)	PCR: *C. baileyi* pos. [46,53] *C. muris* neg. [46,53] oligonuceotide hybridization: *C. baileyi* neg.
Laberge (1996) [29]	a) purified oocysts b) milk	a1–2) 1 oocyst (1.PCR) b1–2) 1–5 oocysts (southern blot and oligonucleotide hybridization)	1) *C. muris* neg. [14,29] *C. baileyi* neg, *C. meleagridis* pos. [14] 2) see Laxer

Continued

Table 3 Continued

Authors	Tested matrices	Reported sensitivity	Crossreaction with other *C.* species
Stinear (1996) [49]	concentrated water samples (spiked with oocysts after concentration procedure)	0.33 oocyst (1.PCR), 0.33 oocyst (southern blot and oligo. hybridization), river and reservoir water concentrates, respectively	*C. muris* and *C. baileyi* negative
Balatbat (1996) [3]	fecal samples	500 oocysts g^{-1} stool (nested PCR), exact number of oocysts/PCR is not calculable from the reported data	see Laxer
Rochelle (1996) [43]	infected cell culture	mRNA from infected cells within 2 h of inoculation with 10^3 oocysts	*C. muris* and *C. baileyi* negative
Bonnin (1996) [5]	purified oocysts	5 oocysts (1.PCR) RFLP analysis was performed with 100–500 oocysts	*C. muris* and *C. baileyi* neg., *C. meleagridis* pos. [14]
Morgan (1996) [37]	a) purified oocysts b) directly from boiled feces	a) 1 oocyst (1.PCR) b) 10^3 g^{-1} stool = 0.03–0.05 oocysts/PCR (1.PCR) 100 oocysts g^{-1} stool = 10 oocysts/PCR (1.PCR)	*C. serpentis* negative [37] *C. muris* and *C. baileyi* neg., *C. meleagridis* pos. [14] see Laxer
Gobet (1997) [22]	fecal samples		
Rochelle (1997) [46]	a) purified oocysts	a1) 10 oocysts (southern blot and oligonucleotide hybridization) a2) 1 oocyst (southern blot and oligonucleotide hybridization) a3) 1 oocyst (second PCR or southern blot and oligonucleotide hybridization) a4) not determined ('inefficient amplification')	1–2) *C. muris* and *C. baileyi* positive 3) *C. muris* and *C. baileyi* negative (see Laxer) 4) *C. muris* negative, *C. baileyi* positive
	b) environmental samples	b1) not determined b2) 50 oocysts after slot blot and oligonucleotide hybridization b3) 500 oocysts after PCR, 5 oocysts after slot blot and oligonucleotide hybridization b4) not determined ('inefficient amplification')	
Patel (1997) [39]	purified oocysts	not determined	*C. muris* negative
Rochelle (1997) [44]	a) purified oocysts b) infected cell culture	a1–2) 1 oocyst (1. PCR) b1) 1 oocyst (mRNA 48 h after cell culture infection), positive by southern blot and oligonucleotide hybridization	1) *C. muris* and *C. baileyi* neg. *C. meleagridis* pos. [14,44] 2) *C. muris* and *C. baileyi* pos.
Spano (1997) [48]	purified oocysts	not determined	differentiation of *C. wrairi* and *C. parvum* by RFLP
Deng (1997) [18]	a) purified oocysts b) fecal samples	a) 10 oocysts (1.PCR and nested PCR) b) 30–100 oocysts (1.PCR)	see Laxer
Carraway (1997) [12]	purified oocysts	not determined	not determined
Wagner-Wiening *et al* (1997) [51]	purified oocysts	not specified	not specified
Rochelle (1997) [45]	not specified	1 oocyst after 48 h of incubation 1000 oocysts after 2 h of incubation	not specified
Chung (1997) [16]	a) purified oocysts b) municipal water sediments	1–5 oocysts per PCR, 1000 oocysts per 10-ml pellet	*C. muris* neg. (see Laberge)
Sluter (1997) [47]	a) purified oocysts and b) lake water sediments	a1–2) 10 oocysts (1.PCR) a3) 100 oocysts (1.PCR) a4) no signal with 1000 oocysts b2) 40 oocysts (1.PCR)	1–3) not determined (modified primers) 4) see Webster 1993
Krüger (1998) [28]	a) purified oocysts b) environmental sediments after IMS	a) 2 oocysts; microscopically verified according to Wiedenmann *et al* b) 10 oocysts	not determined

wrairi [15]. They found that there is crossreaction with this species, which can only be excluded after application of an internal probe. The comparison of the whole amplicon sequences revealed 18–20 bp differences between *C. parvum* and *C. wrairi*, and in addition, gave evidence that the sequence published by Laxer was in error at nine positions. These positions, however, do not affect the primer and probe system. Laberge *et al* [29] report crossreaction of the Laxer primers with *Eimeria acervulina* and crossreaction of one of the probes with *Giardia intestinalis* DNA. The

Table 4 Synopsis of *Cryptosporidium* detection assays: target and sequence information

Authors	Target	Gene function	Multiple copies	Strain differences	GenBank accession
Laxer (1991) [30]	DNA	unclear	unclear	unclear	M59419
Cai (1992) [6]	DNA	18S rRNA, 5.8S rRNA	yes	2 strains, undefined origin	X64340-3
Johnson (1993) [25]	DNA	18S rRNA	yes	unclear	L16996
Ranucci (1993) [40]	DNA	oocyst wall protein	unclear	unclear	Z22537
Webster (1993) [52]	DNA	unclear	unclear	unclear	L01269
Awad-el-Kariem (1994) [2]	DNA	18S rRNA	yes	unclear	X64340-3
Filkorn (1994) [20]	DNA	unclear	unclear	unclear	M59419
Carraway (1994) [10]	DNA	18S rRNA	yes	1 bovine, 1 human/bovine	S76662
Johnson (1995) [26]	DNA	18S rRNA	yes	unclear	L16996
Wagner-Wiening (1995) [50]	DNA	oocyst wall protein	unclear	unclear	M95743
Wiedenmann (1996) [54]	DNA	unclear	unclear	unclear	M59419
Leng (1996) [31]	DNA	unclear	unclear	unclear	M59419
Leng (1996) [32]	DNA	18S rRNA	yes	unclear	L16996
Mayer (1996) [34]	DNA	oocyst wall protein	unclear	unclear	Z22537
Webster (1996) [53]	DNA	unclear	unclear	unclear	L01269
Laberge (1996) [29]	DNA	oocyst wall protein	unclear	unclear	M95743
	DNA	unclear	unclear	unclear	M59419
Stinear (1996) [49]	mRNA	hsp70	yes	unclear	U11761
Balatbat (1996) [3]	DNA	unclear	unclear	unclear	M59419
Rochelle (1996) [43]	mRNA	hsp70	yes	unclear	U11761
Bonnin (1996) [5]	DNA	unclear	yes	1 human, 1 human/bovine	not specified
Morgan (1996) [37]	DNA	unclear	unclear	unclear	not specified
Gobet (1997) [22]	DNA	unclear	unclear	unclear	M59419
Rochelle (1997) [46]	DNA	18S rRNA	yes	unclear	U11761
	DNA	18S rRNA	yes	unclear	U11761
	DNA	unclear	unclear	unclear	M59419
	DNA	unclear	unclear	unclear	L01269
Patel (1997) [39]	DNA	18S rRNA	yes	unclear	L16996
Rochelle (1997) [44]	mRNA	hsp70	yes	unclear	U11761
	DNA	hsp70	unclear	unclear	U11761
Spano (1997) [48]	DNA	oocyst wall protein	unclear	1 human, 1 human/bovine	U35027
Deng (1997) [18]	DNA	unclear	unclear	unclear	M59419
Carraway (1997) [12]	DNA	surface antigen	unclear	2 human, 1 bovine	U83169
Wagner-Wiening (1997) [51]	DNA	not specified			not specified
Rochelle (1997) [45]	not specified				not specified
Chung (1997) [16]	DNA	oocyst wall protein	unclear	unclear	M95743
Sluter (1997) [47]	DNA	unclear	unclear	unclear	M59419
	DNA	18S rRNA	yes	unclear	S76662
	DNA	oocyst wall protein	unclear	unclear	Z22537
	DNA	unclear	unclear	unclear	L01269
Krüger (1998) [28]	DNA	unclear	unclear	unclear	M59419

latter two crossreactions did not occur with their own primer set. On the other hand, the Laberge primers were not checked against *C. wrairi*. This seems to be exemplary for all of the reviewed protocols, as none of them has been evaluated with respect to specificity in the same way, and information, which may appear disadvantageous for one protocol, may simply be lacking for others.

While crossreactions with other genera and species are normally undesirable, they can also be utilized to detect several kinds of organisms at a time in a first step. In a second step, these organisms can be differentiated eg by RFLP. Ho *et al* [24] described a primer pair which amplified a genomic ribosomal sequence of *Neospora*, as well as a similar region in the genome of other genera such as *Toxoplasma* and *Cryptosporidium*.

On the other hand, in certain sequences there is so much variation that it was possible to distinguish two or more different strains of *C. parvum* [5,6,10–12,36,38,48]. One of them could only be found in human isolates [12], which made the authors assume that there is a *C. parvum* subtype which can only infect humans.

Detection of PCR products

PCR and RT-PCR products are usually detected by agarose gel electrophoresis and staining with ethidium bromide. Two relatively new PCR-based methods, which have been applied for *Cryptosporidium* detection, are *in situ* PCR and TaqMan-PCR. For the *in situ* PCR method oocysts [51] or infected cell monolayers [45] are fixed onto microscope slides. After a subsequent permeabilization treatment, the PCR reagents are applied, and the amplification of the target sequence takes place inside of the permeabilized oocysts or cells. A labeled oligonucleotide is hybridized to the amplicon, which can then be detected as an intensively fluorescing, amorphic focus by epifluorescence microscopy. For TaqMan-PCR an additional oligonucleotide probe is added to the reaction mix. This oligonucleotide carries a reporter and a quencher molecule at its ends and specifi-

Table 5 Synopsis of *Cryptosporidium* detection assays: primer sequences, length of target

Authors		Primer sequences (5′ to 3′)	bp
Laxer (1991) [30]	up:	CCG AGT TTG ATC CAA AAA GTT ACG AA	452
	1p:	TAG CTC CTC ATA TGC CTT ATT GAG TA	
	pr1:	CTC AAA GCG AAG ATG ACC TT	
	pr2:	GAA TTA ACC TAT AGG AAC CT	
Cai (1992) [6]	up:	AGT CAT ATG CTT GTC TCA AA	2172
	1p:	TCG CGT TTT GCT GCG TTC TTC	
	pr:	–	
Johnson (1993) [25]	up:	not specified	
	1p:	not specified	
	pr:	not specified	
Ranucci (1993) [40]	up1:	GTC CTA CTG GAT TCA CTC TAC	1130
	up2:	CCA GGA CAT CAT CAT GGT CAT TCT CAT GGG C	753
	1p:	CCG AAT ATG TAA CAC ATT TAT CCG C	
	pr:	–	
Webster (1993) [52]	up:	ATC TTC ACG CAG TGC GTG GT	329
	1p:	CAT CAG CCG GTA GAT GTC GA	
	pr:	ACG TAG CGC CGG ACG ACA GCA CGA GCG CGT	
Awad-el-Kariem (1994) [2]	up1:	AGT GCT TAA AGC AGG CAA CTG	556
	up2:	TAG AGA TTG GAG GTT GTT CCT	
	1p2:	CTC CAC CAA CTA AGA ACG GCC	
	1p1:	CGT TAA CGG AAT TAA CCA GAC	
	pr:	–	
Filkorn (1994) [20]	up:	Laxer (1991)	452
	1p:	Laxer (1991)	
	pr:	–	
Carraway (1994) [10]	up:	not specified	
	1p:	not specified	
	pr:	–	
Johnson (1995) [26]	up:	AAG CTC GTA GTT GGA TTT CTG	435
	1p:	TAA GGT GCT GAA GGA GTA AGG	
	pr:	GGG GAT CGA AGA CGA TCA GAT ACC GTC GTA GTC TTA AC	
Wagner-Wiening (1995) [50]	up1:	AGT GTC CTC CAG GTA CAA ACC TGG TA	873
	up2:	TGC CCA CCT GGA ATA ACA CT	604
	1p2:	TGC CCA TGA GAA TGA CCA TG	
	1p1:	GCA CAG CTG GGA CAG AAT CAG CTT T	
	pr:	–	
Wiedenmann (1996) [54]	up:	Laxer (1991)	452
	1p:	Laxer (1991)	
	pr:	–	
Leng (1996) [31]	up:	Laxer (1991)	452
	1p:	Laxer (1991)	
	pr:	Laxer (1991) pr2	
Leng (1996) [32]	up:	AAC CTG GTT GAT CCT GCC AG	*ca* 1750
	1p:	TGA TCC TTC TGC AGG TTC ACC TA	
	pr:	–	
Mayer (1996) [34]	up1:	Ranucci (1993) up 2	753
	up2:	GGC TCC AAG GCC AAT TTG TG	
	1p2:	GCA TGC CCT GCA GGC TAT GC	
	1p1:	Ranucci (1993) 1p	
	pr:	–	
Webster (1996) [53]	up:	Webster (1993)	329
	1p:	Webster (1993)	
	pr:	–	
Laberge (1996) [29]	1) up:	GCC CAC CTG GAT ATA CAC TTT C	358
	1p:	TCC CCC TCT CTA GTA CCA ACA GGA	
	pr:	GAT CGA TGC TAT CTG CCC AGA TGG A	
	2) up:	Laxer (1991)	452
	1p:	Laxer (1991)	
	pr:	Laxer (1991) pr1	
Stinear (1996) [49]	up:	AGC AAT CCT CTG CCG TAC AGG	590
	1p:	AGA GCA TCC TTG ATC TTC T	
	pr:	–	
Balatbat (1996) [3]	up1:	Laxer (1991) up	452
	up2:	GCG AAG ATG ACC TTT TGA TTT G	194
	1p2:	AGG ATT TCT TCT TCT GAG GTT CC	
	1p1:	Laxer (1991) 1p	
	pr:	Laxer (1991) pr2	

Continued

Table 5 Continued

Authors		Primer sequences (5′ to 3′)	bp
Rochelle (1996) [43]	up:	AAA TGG TGA GCA ATC CTC TG	361
	1p:	CTT GCT GCT CTT ACC AGT AC	
	pr:	CCA TTA TCA CTC GGT TTA GA	
Bonnin (1996) [5]	up:	TTC ATT CTA TCA TGT C	1500
	1p:	ATG GTT ATA TTT GGG	
	pr:	–	
Morgan (1996) [37]	up:	GGT ACT GGA TAG ATA GTG GA	680
	1p:	TCG CAC GCC CGG ATT CTG TA	
	pr:	AGT CCC GTA TCA GTT CGA GA	
Gobet (1997) [22]	up:	Laxer (1991)	452
	1p:	Laxer (1991)	
	pr:	–	
Rochelle (1997) [46]	1) up:	AGT GCT TAA AGC AGG CAA CTG	1) 556
	1p:	CGT TAA CGG AAT TAA CCA GAC	
	pr:	–	
	2) up:	TAG AGA TTG GAG GTT GTT CCT	2) 256
	1p:	CTC CAC CAA CTA AGA ACG GCC	
	pr:	ACG GAA GGG CAC CAC CAG GA	
	3) up:	Laxer (1991)	3) 452
	1p:	Laxer (1991)	
	pr1:	Laxer (1991)	
	pr2:	Laxer (1991)	4) 329
	4) up:	Webster (1993)	
	1p:	Webster (1993)	
	pr:	Webster (1993)	
Patel (1997) [39]	up:	CCG AAT TCG TCG AC [35]	*ca* 1370
	1p:	GTT TAA TAC AGG GAA GTT TTA GGC A	
	pr:	–	
Rochelle (1997) [44]	1) up:	CTG TTG CTT ATG GTG CTG CTG	1) 361
	1p:	CCT CTT GGT GCT GGT GGA ATA	
	pr:	AAA TGG TGA GCA ATC CTC TGC CG	
	2) up:	Rochelle (1996)	2) 307
	1p:	Rochelle (1996)	
	pr:	Rochelle (1996)	
Spano (1997) [48]	up:	GTA GAT AAT GGA AGA GAT TGT G	553
	1p:	GGA CTG AAA TAC AGG CAT TAT CTT G	
	pr:	–	
Deng (1997) [18]	up1:	Laxer (1991)	452
	up2:	GCG AAG ATG ACC TTT TGA TTT G	210
	1p2:	AGG ATT TCT TCT TCT GAG GTT CC	
	1p1:	Laxer (1991)	
	pr:	GAA TTA ACC TAT AGG AAC CT	
Carraway (1997) [12]	1) up1:	TGG TGA ACT GAA GGA TCC	1) 2262
	up2:	CTA CTA CAA CCA AGA AAC C	781
	1p2:	GGT GTA ATG ATT GGA TTA AGA G	
	1p1:	GGG TTC AAG TCA CCA GC	
	2) up:	CTC TTA ATC CAA TCA TTA CAA C	2) 515
	1p:	GAG TCT AAT AAT AAA CCA CTG	
	3) up:	CTC TTA ATC CAA TCA TTA CAA C	3) 318
	1p:	CAG CAA GAT ATG AAT ACC G	
	pr:	–	

Continued

cally anneals to the target sequence between the two primers. The specific fluorescence of the reporter molecule is quenched as long as both molecules are close together. But when the Taq polymerase meets the probe, the exonuclease activity of the polymerase cuts the probe into fragments. The quencher molecule is separated from the reporter and the specific fluorescence of the reporter is no longer suppressed. Thus, the intensity of the fluorescence in the sample increases in the same way as the target sequence is amplified, and finally becomes detectable by a luminescence spectrometer. With novel instruments even an online detection of the increasing fluorescence is possible, and the time when the signal exceeds a threshold value can be used to estimate the number of target copies, which were present in the sample at the beginning of the reaction. TaqMan-PCR for *Cryptosporidium* detection is currently evaluated in the authors' lab and has been included in this review. The sensitivity of this method seems to be comparable to the sensitivity of a conventional PCR with product detection in agarose gels after ethidium bromide staining [28].

Table 5 Continued

Authors		Primer sequences (5′ to 3′)	bp
Wagner-Wiening (1997) [51]		not specified	
Rochelle (1997) [45]		not specified	
Chung (1997) [16]	1) up:	CCG AGT TTG ATC CAA AAA GTT AC (Laxer, 1991, modif.)	1) 449
	1p:	GCT CCT CAT ATG CCT TAT TGA G (Laxer, 1991, modif.)	
	pr:	–	
	2) up:	GCG AAT TCC TGA CAC AGG GAG GTA G (Carraway, 1996, modif.)	2) 506
	1p:	GCG GGA TCC TTG GCA AAT GCT TTC G (Carraway, 1996, modif.)	
	pr:	–	
	3) up:	GTC CTA CTG GAT TCA CTC TA (Ranucci, 1993, modif.)	3) 1130
	1p:	GAA TAT GTA ACA CAT TTA TCC GC (Ranucci, 1993, modif.)	
	pr:	–	
	4) up:	Webster (1993)	4) 329
	1p:	Webster (1993)	
	pr:	–	
Sluter (1997) [47]	up:	GCC CAC CTG GAT ATA CAC TTT C	358
	1p:	TCC CCC TCT CTA GTA CCA ACA GGA	(IS) 225
	pr:	–	
Krüger (1998) [28]	up:	CCT TTT GTA GCT CCT CAT ATG CCT TA (rcf!)	179
	1p:	ACT TCA CGT GTG TTT GCC AAT G (rcf!)	
	pr:	TCA AAC GCT TCT CTA GCC TTT CAT GAC TTG TCT (rcf!)	

IS = Internal Standard.
rcf = reverse complemented form.
up = upper primer.
lp = lower primer.
pr = probe.
bp = base pairs.

Enhancement of specificity

Hybridization of PCR products with specific oligonucleotide probes, as in TaqMan-PCR and *in situ* PCR, provides an additional specificity control. For conventional PCR, a similar effect can be achieved by performing semi-nested or nested PCR, by oligonucleotide hybridization after a dot blot, slot blot or southern blot or by restriction fragment analysis of the amplicon.

Tested matrices

To establish and optimize their PCR-based detection methods, all authors have used oocyst preparations, which had been purified from stool samples of naturally or artificially infected animals or human beings. Only some of them have evaluated their methods for environmental or food matrices [16,25,26,28,29,34,46,47,49], and none of the methods has been standardized to such an extent that it was tested in interlaboratory studies for reproducibility and repeatability.

Sensitivity

One of the most important features of a PCR-based detection system for water and food analysis is certainly its sensitivity. As single organisms can be detected by microscopy, a system which is intended to replace microscopy, should ideally detect one oocyst, also. For a PCR method this does not seem to be a completely unrealistic goal for several reasons: theoretically, a single copy of a target sequence can be sufficient to start the amplification process. As *Cryptosporidium* oocysts always contain four sporozoites, even single copy gene sequences are always present four times. If multiple copy DNA sequences, rRNA or mRNA are used as a target, single oocysts should normally contain enough material to start a PCR. According to Guay *et al* [23] there are probably >100 copies of the rRNA gene present in the eucaryotic genome.

Single oocyst detection has been reported by several authors and for different PCR systems [26,29,31,37,44–46,49,55]. It has, however, to be emphasized that sensitivity testing has always been done with dilution series of either oocyst suspensions, sporozoite suspensions or isolated nucleic acids, so it was never proven that really only one single oocyte was used. This may explain the somewhat striking fact, that in some publications the sensitivity was reported or could be calculated to be less than one oocyst or even less than one sporozoite [26,31,37,49,53]. This problem has been addressed by Wiedenmann *et al* [55], who have described a method, which allows exact identification of the number of oocysts by epifluorescence microscopy, before the very same oocysts are subjected to the PCR assay. Using this procedure, the detection of two microscopically verified oocysts in four out of 10 consecutive experiments has been reported [28]. These experiments have confirmed the applicability of this method and gave additional evidence, that single oocyst detection by PCR is possible.

Quantification

Most of the described PCR assays are presence-absence tests. Though under laboratory conditions the signal strength approximately corresponds to the number of target sequence copies in the reaction mix, there is general agreement that in real samples the signal strength in itself is no reliable means for quantification. One possibility of getting quantitative or at least semi-quantitative PCR results is to introduce an internal standard (IS) into the reaction. The

standard is an oligonucleotide, which is added in constant low concentration to every reaction mix. It is amplified by the same primers as the wild-type target (WT), but produces an amplicon with different length. The strength of the IS-signal decreases with growing amounts of start copies of the WT sequence in the sample. The WT/IS relation can then be used to generate a standard curve, which allows a more precise quantification than the analysis of the WT signal alone. Chung *et al* [16] adapted this procedure to a commercially available detection system for PCR amplification products (Digene Sharp Signal™ assay). This assay transforms the amount of PCR product into an equivalent signal for colorimetric detection in a microplate reader, and the WT/IS absorbance relation has been used to quantify the results. Another approach for quantification could be a most probable number technique. This would, however, require bigger sample volumes and multiple sample preparation steps, and none of the published protocols has yet been used in this way. As already explained, TaqMan-PCR with real time detection of fluorescence may provide another possibility to get quantitative PCR results.

Synopsis of reviewed methods

Tables 1–5 provide a synopsis of the reviewed methods by specifying the following components: isolation and purification of oocysts from tested matrices, elimination of free DNA, viability assessment, release of nucleic acids, nucleic acid extraction, target information, sequence information, type of PCR, product detection, additional specificity control, secondary product detection, tested matrices, reported sensitivity, and crossreaction with other *Cryptosporidium* species.

We have tried to include in these tables all available protocols which have been suggested or have been used for PCR detection of *Cryptosporidium* oocysts in environmental samples. The tables do not include various PCR protocols which have been described only for *Cryptosporidium* detection in patients or tissues and which have only been used to detect strain differences or to perform genetic analyses.

Conclusions and outlook

Since 1991, when the first PCR protocol for the detection of *C. parvum* was published by Laxer *et al* [30], an exponentially growing number of publications has dealt with this topic. The improvements which have been made since then comprise: (a) the identification and the removal of PCR inhibitors from environmental samples by novel separation techniques like IMS, and by efficient isolation methods for nucleic acids; (b) the development of at least two different ways for the assessment of oocyst viability, either by combination with *in vitro* excystation or by detection of mRNA; (c) the development of infectivity assays, which combine *in vitro* excystation with cell culture infection; and (d) an increase in sensitivity, which makes the PCR methods comparable to the sensitivity of conventional microscopic immunofluorescence methods.

Systematic comparisons of PCR methods with conventional microscopy, including interlaboratory studies, are to be expected in the near future, and there is already enough evidence to assume that epidemic levels of 10–30 oocysts per 100 liters of finished drinking water could be reliably detected with present PCR methods. Exact quantification by diagnostic PCR assays is still a problem. Application of internal standards, *in situ* PCR and TaqMan-PCR may provide at least semi-quantitative results and are currently under development or evaluation. It is, however, free of doubt that the most beneficial use of diagnostic PCR methods could be made, if presence-absence standards for water, food and environmental matrices would be set, eg '*Cryptosporidium parvum* not detectable in certain matrix volumes'. New cycler generations, which shorten the time required for 30 cycles to no more than 30 min, will further contribute to the applicability of PCR methods, and make PCR results available within 1 working day, even when additional time-consuming procedures like sample preparation, viability assays, nested PCRs etc have to be performed. Several PCR protocols are capable of detecting strain differences within human and between human and bovine stool isolates of *C. parvum* [5,6,10–12,36,38,48]. It seems possible that this capability can be combined with highly sensitive diagnostic methods for environmental and food samples, and thus provide a tool for epidemiologic studies. PCR in conjunction with viability or cell culture infectivity assays may be an attractive way to assess the effect of disinfection procedures and it should be evaluated whether they are a suitable alternative to animal models. The problem of crossreaction with other *Cryptosporidium* species has not yet been addressed for all existing protocols in a uniform way. The only recently reported lack of specificity of eight different primer systems, when compared to *C. meleagridis* [14], may be looked upon as a provisional drawback. However, the present microscopic immunofluorescent method also lacks species specificity, and it seems to be at least as likely that specific primers can be found, as that specific antibodies can be generated in the future.

Acknowledgements

The results in Tables 1–5, which refer to TaqMan-PCR were generated under contract No. 11163 of the Deutsche Bundesstiftung Umwelt (DBU), Osnabrück, Germany and with Anti-*Cryptosporidium* IMS kits donated by Deutsche Dynal GmbH, Hamburg, Germany.

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