A simple and efficient transfection protocol for *Cryptosporidium parvum* using Polyethylenimine (PEI) and Octaarginine

Tran Nguyen-Ho-Bao1,2, Maxi Berberich1, Wampeng Zheng1, Dieter Seebach3, Arwid Daugschies1 and Faustin Kamena1,4

1Faculty of Veterinary Medicine, Institute of Parasitology, Centre for Infectious Medicine, University of Leipzig, 04103 Leipzig, Germany; 2Department of Veterinary Medicine, College of Agriculture, Can Tho University, 900000 Can Tho, Vietnam; 3Laboratory for Organic Chemistry, Swiss Federal Institute of Technology, ETH-Z, Zurich, Switzerland and 4Department of Microbiology and Parasitology, Laboratory for Molecular Parasitology, University of Buea, Cameroon, PO Box 63, Buea, Cameroon

Abstract

The transfection of *Cryptosporidium* represents a major challenge, and current protocols are based on electroporation of freshly excysted sporozoites using a rather large amount of plasmid DNA which typically has a very poor yield. In this study, we report a fast and simple protocol for transfection of *Cryptosporidium parvum* that takes advantage of the DNA condensing power of the poly cationic polymer polyethylenimine (PEI) and the gene delivery property of the short cell-penetrating peptide octaarginine. Our novel protocol requires a very low amount of plasmid DNA and does not necessitate special laboratory equipment to be performed. Transfection appears to be more efficient in oocysts just triggered for excystation than the excysted sporozoites. Altogether, the application of octaarginine with PEI allows efficient transfection. To the best of our knowledge, this is the first report on an electroporation-free protocol for transfection of sporozoites of a *Cryptosporidium* species.

Introduction

*Cryptosporidium parvum* is a small intracellular parasite that infects a wide range of mammalian hosts, including humans and calves. The introduction of highly active anti-retroviral therapy (HAART) against HIV infection has significantly reduced the prevalence of severe human cases of *Cryptosporidium* infection in industrialized countries (Ives *et al*., 2001). However, in developing countries, cryptosporidiosis remains a major life-threat to young children (Ventura *et al*., 2013). The only approved drug to treat human cryptosporidiosis, nitazoxanide, shows poor efficacy in immunocompromised patients and malnourished children (Manjunatha *et al*., 2017). Novel or alternative treatment strategies are desperately needed; however, no major breakthrough has been achieved so far. Compared to other apicomplexan parasites, *Cryptosporidium* research is still at its infancy. Particularly the development of tools to edit the parasite genome has been a major frustration point and the transfection of *Cryptosporidium* is still a major challenge. Current protocols are based on electroporation of freshly excysted sporozoites using a rather large amount of plasmid DNA which typically results in a very poor yield of transfected parasites.

The poly cationic polymer polyethyleneimine (PEI) with its tertiary amines possesses remarkable DNA condensing properties but can also provide essential buffering capacities that enable an efficient endosomal escape and nucleus penetration (Boussif *et al*., 1995; Fischer *et al*., 1999). However, due to its cellular toxicity, PEI has not been widely used in research (Florea *et al*., 2002; Moghimi *et al*., 2005). Importantly, it has been reported that combining PEI with cell-penetrating peptides (CPPs) significantly reduces its cytotoxic effect while the gene delivery efficiency is increased (Kilk *et al*., 2005; Munyendo *et al*., 2012; Yang *et al*., 2019). The small peptide octaarginine possesses known cell-penetrating properties and is known to cross all known biological membranes. Herein we report a simple and fast transfection protocol that combines the properties of the two molecules to achieve the transfection of *Cryptosporidium* with a minimal amount of DNA. The novel protocol does not require electroporation and works best with oocysts immediately after initiation of excystation rather than with excysted sporozoites.

Materials and methods

Preparation and excystation of *C. parvum* sporozoites

The house strain *Cryptosporidium parvum* (GP-60 subtype Ila, A15G2RI) was originally isolated from a commercial cattle farm (Wilchwitz, Germany in 2015). For propagation, neonate calves were orally infected with $9 \times 10^7$ oocysts. Oocysts were collected from the feces 4–13 days post infection, cleaned and isolated following the protocol described by Najdrowski *et al*. (2007). Subsequently, cleaned oocysts were stored in PBS pH = 7.2 (Gibco®, ThermoFisher Scientific, Massachusetts, USA) supplemented with penicilline/streptomycine (200 μg mL$^{-1}$) and...
amphotericin B (5 μg mL\(^{-1}\)) at 4°C for up to 3 months before using. The PBS pH 7.2 described above was used throughout this study and will be referred to as PBS in the remaining text. The storage medium was changed monthly to prevent bacterial and fungal contamination. Before excystation, oocysts were bleach with 5.25% sodium hypochlorite in PBS in the ratio 1:1 then put on ice for 5 min. Decontaminated oocysts were washed 3 times with cold PBS and resuspended in excystation medium (0.8% sodium taurocholate - NaT in the DMEM with 2% fetal calf serum, 1% amphotericin B and 1% penicillin/streptomycin, 1% sodium pyruvate) and pre-incubated at 15°C for 10 min, then further incubated at 37°C and 5% CO\(_2\) for 3 h. Sporozoites were centrifuged for 5 min at 9,500 × g (Centrifuge MIKRO 200, Merck, Darmstadt, Germany) to remove excystation medium and then resuspended in the infection medium (DMEM with 2% fetal calf serum, 1% amphotericin B and 1% penicillin/streptomycin, 1% sodium pyruvate).

**Preparation of short time excystation exposed (STE) oocysts**

STE oocysts were obtained through a similar procedure which was described in the preparation of *C. parvum* sporozoites. However, the incubation step at 37°C in the presence of excystation medium was reduced to only 15 min.

**Synthesis of 6-Fam-labelled octaarginine**

Octaarginine labelled with 6-carboxyfluorescein (R8-FAM) was synthesized as described in (Sparr et al., 2013).

**Uptake of 6-Fam-labelled octaarginine and direct fluorescence microscopy**

6-Fam-labelled octaarginine (10 μg mL\(^{-1}\)) was added to either intact oocysts, STE oocysts or sporozoites of *C. parvum*, followed by further incubation in infection medium (DMEM with 2% fetal calf serum, 1% amphotericin B and 1% penicillin/streptomycin, 1% sodium pyruvate) for 30 min at 37°C. Intracellular 6-Fam-labelled octaarginine was visualized microscopically by direct fluorescence using a Leica TCS SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany). Briefly, parasites were centrifuged for 5 min at 9,500 × g, then washed with PBS 3 times. Parasites were then fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, followed by washing 3 times with PBS. Next, 4,6-diamidino-2-phenylindole (DAPI; 10 μg mL\(^{-1}\)) was added and incubated for another 10 min at room temperature. After a final extensive washing 3 times with PBS, parasites were mounted on glass slides using fluoromount-G (Southern Biotech, Birmingham, USA).

**DNA condensation by PEI/octaarginine and gel retardation assay**

Branchied PEI (60 kDa), (Sigma-Aldrich, Hamburg, Germany) was dissolved in distilled water to prepare a stock solution of 10 mg mL\(^{-1}\). PEI was passed through a 0.2 μm filter (Millipore®, Merck, Darmstadt, Germany) and stored at −20°C until use. For these experiments, we used the GFP-expressing plasmid Eno_prom_GFP_neo_eno_utr. This is a plasmid using an enolase promoter and containing a neomycin resistance gene. Various amounts of DNA/PEI were combined to form complexes with plasmid DNA concentrations ranging from 1 μg to 20 μg and having different N/P ratios where N represents the number of moles of amine nitrogen in PEI and P is the number of moles of phosphate in the DNA. The calculation for the ratio N/P was done as described by Boussif et al. (1995). Briefly, given the molecular weight of a nucleotide of 330 g/mol, 10 μg of plasmid represent 30 nmol. In order to obtain an N/P ratio of 10, the concentration of PEI needed is 300 nmol which represents 1.3 μL from the 10 mg mL\(^{-1}\) stock solution. For gel retardation assay, solutions of DNA/PEI or DNA/PEI plus octaarginine (in 10-fold excess) were prepared and incubated at 37°C for 30 min, in order to allow DNA condensation. The complexes of DNA/PEI and DNA/PEI with octaarginine were separated on 0.9% agarose gel electrophoresis for 45 min and 120 V. GeneRuler 1 kb DNA Ladder® (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used as a reference. The complexes were visualized by staining with 0.01% ethidium bromide for 30 min. The gels were visualized under UV light using the bio-imaging system ArgusX1 (Biostep, Burkhardsdorf, Germany).

**Transfection of sporozoites, STE oocysts and oocysts**

Complexes of the plasmid with either PEI alone or PEI and octaarginine were incubated with 2 × 10⁵ freshly excysted *C. parvum* sporozoites, intact oocysts or STE oocysts at 37°C for 1 h in PBS. Excess reagent was removed by centrifugation for 1 min at 9,500 × g. Parasites were resuspended in infection medium and added to confluent HCT-8 cell cultures.

**Culture of HCT-8 cells and infection**

For infection experiments, host cells were cultured as described by Najdrowski et al. (2007) with some modifications. Briefly, human ileocecal carcinoma cells (HCT-8) were seeded in 24-well plates containing a microscopy slide at the bottom and cultured in RPMI-1640 supplemented with 10% fetal calf serum (Northumbria, Cramlington, UK), antibiotics (1% penicillin/streptomycin, and 1% amphotericin B) and 1% sodium pyruvate incubated at 37°C with 5% CO\(_2\). Cells were infected with 70% confluence with 2 × 10⁵ freshly transfected sporozoites or STE oocysts (as described above) in infection medium and incubated for either 24 or 48 h.

**Immunofluorescence assay**

For the immunofluorescence assay, HCT-8 cells infected with transfected sporozoites were fixed (4% paraformaldehyde) for 20 min at room temperature and permeabilized (0.2% Triton X-100 in PBS) for 20 min at room temperature. Before visualization, coverslips were washed with PBS, diamidino-2-phenylindole (DAPI; 10 μg mL\(^{-1}\)) was added and the slides were further incubated for 10 min at room temperature. After a final extensive washing 3 times with PBS, parasites were mounted on glass slides using fluoromount-G (Southern Biotech, Birmingham, USA).

**Results**

**Permeability of *C. parvum* membranes to octaarginine**

Before applying PEI and octaarginine as transfection agents for *Cryptosporidium* we first aimed as assessing the permeability of
the parasite membrane for CPPs. For this reason, fluorescently labelled octaarginine (R8-FAM) was incubated with freshly excysted *C. parvum* sporozoites, intact oocysts, or STE oocysts and peptide uptake was monitored using confocal microscopy. We found that the membrane of intact oocysts was impermeable to octaarginine as shown by the green fluorescence staining only the cell wall of the oocysts (Fig. 1A). In contrast, free sporozoites, as well as STE oocysts, were able to take up the peptide rather rapidly (Fig. 1B and C).

**Condensation of plasmid DNA with PEI and formation of the transfection complex**

As next, we decided to investigate the optimal condition for condensing plasmid DNA with PEI and octaarginine using the gel retardation assay. For this purpose, we applied several combinations of PEI and plasmid DNA in order to identify the most suitable **N**/**P** ratio for full condensation of DNA where **N** represents the number of charged amine functions and **P** the number of charged phosphate groups of the DNA backbone. Typically, the optimal **N**/**P** ratio allows the total condensation of the DNA available. Using 5 μg plasmid DNA as starting point and combining with PEI we tested the following **N**/**P** ratios 2, 4, 6, 8, 12, 16 and 20 and observed that with an **N**/**P** ratio of 12 or more, the entire plasmid was condensed (Fig. 2A). In the next step, we added octaarginine to the generated DNA/PEI complex in order to form the final transfection complex. Interestingly, when octaarginine was added to the DNA/PEI complex the **N**/**P** ratio required to achieve full condensation appeared to be slightly lower (8) than when PEI alone (12) was used, as seen on the retardation assay (Fig. 2B).

**Transfection of C. parvum with the DNA/PEI/octaarginine complex**

Next, we decided to assess whether the transfection complex could be successfully transferred to the parasite without electroporation. For this purpose, we used freshly excysted and purified sporozoites, STE oocysts as well as intact oocysts. The transfection complex consisting of plasmid DNA, PEI and octaarginine was incubated with each of the above-mentioned parasite materials for 1 hour at 37°C. Thereafter, these parasites were transferred to an exponentially growing culture of HCT-8 cells. Successful transfection was assessed by confocal microscopy after 24 h. We found, as expected, that with intact oocysts, no transfection could be observed (data not shown). This is in accordance with the impermeability of intact oocyst membranes to octaarginine as demonstrated with the fluorescently labelled peptide (Fig. 1A). Using excysted sporozoites, transfected parasites expressing the green fluorescent protein could be observed (Fig. 3A). However, the overall infection rate of transfected sporozoites was rather low and infected host cells could only be found sporadically. When STE oocysts were used, a higher infection rate was observed and transfected parasites in various developmental stages could be visualized (Fig. 3B). Importantly, when STE oocysts were transfected using only 1 μg of plasmid DNA we were also able to see few GFP-expressing parasites (Fig. 3C). Transfection with either PEI and plasmid or octaarginine and plasmid did not yield any GFP expressing parasite (data not shown).

**Discussion**

It has been shown in the past that polymers with a high density of primary amine functions have the ability to facilitate gene delivery...
Because of its excellent DNA condensing properties, PEI is an attractive candidate for transfection. However, its rather high molecular size brings toxicity issues for the host cells (Nam et al., 2015) or decreases the cell metabolic activity (Fischer et al., 1999; Godbey et al., 2001; Florea et al., 2002). Although a CPP alone is unable to properly condense DNA plasmid (Jeong et al., 2009). (Ou et al., 2009).
et al., 2016), the combination of CPPs and PEI significantly improves the transfection efficiency. Moreover, some CPPs namely octaarginine can also inhibit the intracellular proteolytic systems like the proteasome (Klöf et al., 2009) but it is unknown whether they can permanently inhibit cellular function. Certain CPPs can safely deliver their cargos such as DNA plasmid, therapeutics into the cells and are applied in in vitro and in vivo studies. For instance, when Glycosaminoglycan (GAG)-enhanced transduction (GET) peptides were combined to PEI, the overall efficiency of transfection of nanoparticles for lung gene therapy was significantly improved in the mouse model (Osman et al., 2018).

Importantly, the ability of octaarginine to deliver a cargo to a given compartment is not limited by the size of the corresponding cargo but rather by the membrane permeability of the corresponding compartment to octaarginine. Almost all biological membranes tested so far seem permeable to octaarginine with the only exceptions recorded so far being the membranes of non-infected red blood cells and the parasitophorous vacuole membrane of T. gondii (Kamen et al., 2011; Sparr et al., 2013). Our observation that the membrane of intact oocysts is completely impermeable to octaarginine while STE oocysts and free sporozoites efficiently take up the peptide, suggests that free sporozoites and STE oocysts would be more appropriate for plasmid delivery using the polycationic molecules.

In addition, our findings showed that the combination of PEI with octaarginine improve the DNA condensation thereby facilitating the plasmid delivery to Cryptosporidium for parasite transfection. Although a covalent conjugation of a cell-penetrating peptide to PEI seems to be more efficient for the delivery process, a non-covalent complex as applied in our experiments is also capable of delivering DNA to the cell (Kilk et al., 2005). More importantly, the use of a non-covalent complex represents an easy to use alternative that does not require any specific chemical modification of the compounds involved and as such will be accessible to a wider range of scientists. It was reported previously that the use of a CPP alone is not sufficient for efficient plasmid DNA condensation (Kilk et al., 2005) and although TP10 and not octaarginine was the CPP used in that report it is very likely that octaarginine will display the same properties based on their structural similarities. The positive effect on transfection observed in this study is really the result of the combination of the two polycationic molecules PEI and octaarginine.

In conclusion, we have developed a simple protocol for the transfection of Cryptosporidium parvum. The novel protocol takes advantage of the DNA condensing properties of PEI and the cell-penetrating properties of octaarginine. The combination of these two poly cationic molecules seems optimal for the delivery of plasmid DNA into Cryptosporidium. Importantly, successful transfection depends on the permeability of the parasite membrane to octaarginine albeit not exclusively as free sporozoites, although they can rapidly take up octaarginine, were unable to efficiently support transfection. This is probably due to the rapid loss of infectivity during the various incubation steps. Intact oocysts are totally impermeable to octaarginine and hence do not allow any plasmid uptake. In contrast, when oocysts are pre-incubated with excystation agents for a short time that does not allow complete excystation (STE oocysts), their wall is obviously less resistant to penetration by the transfection complex. Accordingly, successful transfection of the sporozoites inside these oocysts was demonstrated. It is plausible to think that sporozoites retain infectivity for a longer period if they remain inside the oocysts in comparison to fully excysted sporozoites. Therefore, preservation of infectivity is an essential factor in the whole transfection process. A major advantage of our protocol is the fact that it works without electroporation and does not require any other sophisticated equipment. It is thus much easier to perform, affordable and applicable by a wider range of scientists around the world.

Acknowledgement. We acknowledge the Biologging Core Facility Universität Leipzig, Germany for enabling confocal microscopy.

Financial support. The work was supported by a doctoral scholarship of the Vietnamese Government (Project 911) to Tran Nguyen-Ho-Bao.

Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. All animal work was conducted in accordance with the German ‘Tierschutzgesetz in der Fassung vom 22. Juli 2009,’ which implements Directive 2010/63/EU from the European Parliament and Council (On the Protection of Animals Used for Scientific Purposes). The protocol was approved by the Saxon federal authorities.

References

Boussif O, Lezoualch’t F, Antoniet ZM, Mergny MD, Scherman D, Demeneix B and Berh JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences of the Unites States of America—Biochemistry 92, 7297–7301.

Fischer D, Bieber T, Li Y, Elsäßer HP and Kissel T (1999) A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethyleneimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharmaceutical Research 16, 1273–1279.

Florea BL, Meaney C, Junginger HE and Borchard G (2002) Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. The American Association of Pharmaceutical Scientists 4, 1–11. https://doi.org/10.1208/ps040312

Godfrey WT, Wu KK and Mikos AG (2001) Poly(ethyleneimine)-mediated gene delivery affects endothelial cell function and viability. Biomaterials 22, 471–480.

Ives NJ, Gazzard BG and Easterbrook PJ (2001) The changing pattern of AIDS-defining illnesses with the introduction of highly active antiretroviral therapy (HAART) in London clinic. Journal of Infection 42, 134–139. https://doi.org/10.1053/jinf.2001.0810

Jeong C, Yoo J, Lee DY and Kim YC (2016) A branched Tat cell-penetrating peptide as a novel delivery carrier for the efficient gene transfection. Biomaterials Research 20, 1–8.

Kamen F, Monnanda B, Makou D, Capone S, Patora-Komisarska K and Seebach D (2011) On the mechanism of eukaryotic cell penetration by α- and β-oligoarginines – targeting infected erythrocytes. Chemistry and Biodiversity 8, 1–12.

Kilk K, El-Andaloussi S, Jërder P, Meikas A, Valkna A, Bartfai T and Langel Ü (2005) Evaluation of transportan 10 in PEI mediated plasmid delivery assay. Journal of Controlled Release 103, 511–523.

Klöf A, Henklein P, Siele D, Schmolke M, Apcher S, Kuchn L and Dahmann B (2009) The cell-penetrating peptide octa-arginine is a potent inhibitor of proteasome activities. European Journal of Pharmacology and Biopharmaceutics 72, 219–225.

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Pangchalingam S, Wo Y, Sow SO, Sur D, Breiman RF, Faruque ASG, Zaidi AKM, Saha D, Alonso PL, Tamboura B, Sanogo D, Owuchekwu U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hassain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hassain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O’Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM and Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. The Lancet 382, 209–222.

Manjunatha UH, Vinayak S, Zambriski JA, Chao AT, Sy T, Noble CG, Bonamy GMC, Kondreddi RR, Zo B, Gredek P, Brooks CE, Herbert GT, Sateriale A, Tandel J, Noh S, Lakshminarayana SB, Lim SH, Goodman LB, Feng G, Zhang L, Blasco F, Wagner J, Joel F, Streepen B and Diagana TT (2017). A Cryptosporidium Pl (4) K inhibitor is a drug
candidate for cryptosporidiosis. *Nature* **546**(7658), 376–380. https://doi.org/10.1038/nature22337.

Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G and Szewczyk A (2005) A two-stage poly(ethyleneimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Molecular Therapy* **11**, 990–995.

Munyendo WLL, Lv H, Benza-Ingoula H, Baraza LD and Zhou J (2012) Cell penetrating peptides in the delivery of biopharmaceuticals. *Biomolecules* **2**, 187–202.

Najdrowski M, Heckerth AR, Wackwitz C, Gawłowska S, Mackenstedt U, Kliemt D and Daugschies A (2007) Development and validation of a cell culture based assay for in vitro assessment of anticryptosporidial compounds. *Parasitology Research* **101**, 161–167.

Nam K, Jung S, Nam J-P and Kim SW (2015) Poly(ethyleneimine) conjugated bioreducible dendrimer for efficient gene delivery. *J Control Release* **220**, 447–455.

Osman G, Rodriguez J, Chan SY, Chisholm J, Duncan G, Kim N, Tatler AL, Shakesheff KM, Hanes J, Suk JS and Dixon JE (2018) PEGylated enhanced cell penetrating peptide nanoparticles for lung gene therapy. *Journal of Controlled Release* **285**, 35–45. https://doi.org/10.1016/j.jconrel.2018.07.001.

Ou M, Xu R, Kim SH, Bull DA and Kim SW (2009) A family of bioreducible Poly (disulfide amines) for gene therapy delivery. *Biomaterials* **30**, 5804–5814.

Sparr C, Purkayastha N, Kolesinska B, Gengenbacher M, Amulic B and Matuschewski K (2013) Improve efficacy of Fosmidomycin against Plasmodium and Mycobacterium species by combination with the cell-penetrating peptide octaarginine. *Antimicrob Agents Chemotherapy* **57**, 4689–4698.

Ventura G, Cauda R, Larocca LM, Riccioni ME, Tumbarello M and Lucia MB (1997) Gastric cryptosporidiosis complicating HIV infection: case report and review of the literature. *European Journal of Gastroenterology & Hepatology* **9**, 307–310.

Yang S, Wang D, Sun Y and Zheng B (2019) Delivery of antisense oligonucleotide using polyethylenimine-based lipid nanoparticle modified with cell penetrating peptide. *Drug Delivery* **26**, 965–974.