Analysis of *Cryptosporidium spp.* FromClinical Samples by Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry

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Abbreviated running headline not exceeding 35 letters and spaces: Cryptosporidium and MALDI-TOF

# **Abstract**

Aim: To purify *Cryptosporidium* spp.oocysts from clinical stool samples and evaluate using an up-to-date mass spectrometry protocol producing high quality reference spectra.

Methods and Results: A refined purification protocol was developed for oocysts from stools, involving salt flotation and potassium bromide density centrifugation. Purified oocysts were prepared for matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry (MALDI-TOF MS) by formic acid extraction and the extracts analysed using the Bruker MALDI Biotyper System. Individual spectral markers were identified by their specific mass peaks. *Cryptosporidium parvum* oocysts (Iowa strain) propagated *in vivo*, and *C. parvum* (n=2) and *Cryptosporidium hominis* (n=1) oocysts from clinical stool samples, produced distinct spectra that were considered specific to *Cryptosporidium* spp. with no evidence of contamination.

Conclusions: The production of distinct spectra demonstrated the utility of the purification method for oocysts from clinical stool samples and provided reference spectra.

Significance and Impact of Study: The use of MALDI-TOF MS and other mass spectrometry techniques has been limited previously to *C. parvum* oocysts propagated *in vivo*. Appropriate purification of oocysts can achieve sufficient biomass enabling analysis by MALDI-TOF MS and potentially other mass spectrometry platforms, facilitating peptide and protein discovery and identification of biomarkers from a much wider range of *Cryptosporidium* spp. from natural infections.

**Key words:** Cryptosporidium, purification, matrix-assisted laser desorption / ionisation time-of-flight, MALDI-TOF, mass spectrometry

# **Introduction**

*Cryptosporidium* is a genus of apicomplexan parasites that can cause life-threatening gastroenteritis (cryptosporidiosis) in young children and immunocompromised individuals worldwide; it has been estimated to be responsible for over 57000 deaths in 2016, with over 80 % of those among children under five years old (Khalil *et al.* 2018). The parasite is highly transmissible through person-to-person or animal-to-person spread, and through food, water or the environment. Currently, specific therapy is limited and there are no vaccines against cryptosporidiosis which presents difficulties for the prevention and treatment of the disease (Ryan *et al.* 2016). Anti-*Cryptosporidium* vaccine design programmes are underway and it is clear that further insight into host-parasite interactions will be crucial for these developments (Checkley *et al.* 2015; Ryan *et al.* 2016).

 Two *Cryptosporidium* species predominate in human infection: *Cryptosporidium parvum* which has a wide host range, and *Cryptosporidium hominis* which is host-adapted to humans. Other species more usually found in animals have also been implicated in human infection. Proteomic studies have been limited for *Cryptosporidium* spp. by the requirement for high numbers of purified parasites; the transmissive oocyst stage containing infective sporozoites is shed in faeces which challenges preparations. In addition, it is important that extracts for mass spectrometry are devoid of residues that would cause an unexpected decrease in signal on the final spectra due to the formation of cation adducts (Matsubayashi *et al.* 2005). Investigating the proteome of *Cryptosporidium* spp. supplements the current advances in genomics by facilitating the identification of post-translational modifications and other proteolytic events (Barrett *et al.* 2000; Sanderson *et al.* 2008), leading to greater understanding of infection and disease initiated by sporozoites (Jean Beltran *et al.* 2017). For example, traceable investigation of the life cycle development has recently been enabled by infecting cells with genetically engineered strains (Tandel *et al.* 2019). Additionally, profiling the oocyst proteome of different species and subtypes could help to identify unique or recurrent proteins as diagnostic biomarkers (Crutchfield *et al.* 2016).

 Troung and Ferrari used flow cytometry and 2D-PAGE to evaluate the purity of oocyst preparations from calf faeces using sucrose-Percoll® gradients (Truong and Ferrari, 2006). Additionally, mass spectra need to be investigated for potential contaminants. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is widely available in clinical laboratories and could potentially provide mass spectra leading to protein identification and peptide mass fingerprints (Siddiki, 2013).

 A literature search of PubMed and Scopus using the search terms “*Cryptosporidium* AND MALDI” identified six relevant studies. In early proof of principle studies, mass spectra were produced for *C. parvum* (Glassmeyer *et al.* 2007) and for *C. parvum* and *C. muris* (Magnuson *et al.* 2000). In more recently published studies further analysis of peptides and antigens following digestion of proteins extracted from whole or excysted oocysts and exposed sporozoites was reported (Sanderson *et al.* 2008; Lee *et al.* 2011; Matsubayashi *et al.* 2013; Haserick *et al.* 2017). However, all these studies used experimentally propagated oocysts; four used the *C. parvum* Iowa “strain” propagated in calves and/or mice or lambs (Magnuson *et al.* 2000; Glassmeyer *et al.* 2007; Sanderson *et al.* 2008; Haserick *et al.* 2017) and two used other isolates propagated in mice (Lee *et al.* 2011; Matsubayashi *et al.* 2013). While animal propagation provides the large numbers of oocysts (biomass) required to mitigate against substantial losses during purification (Magnuson *et al.* 2000), it is not ethically sustainable. Furthermore, there is a paucity of peptide and protein data for different *C. parvum* isolates, and for other *Cryptosporidium* species such as *C. hominis* that lack readily available animal models. Widening the literature search to other mass spectrometry platforms revealed a similar scarcity of studies and ensuing lack of database content (see for example <https://cryptodb.org/cryptodb/showQuestion.do?questionFullName=GeneQuestions.GenesByMassSpec> or <http://www.fiserlab.org/epicdb>). While the development of continuous culture systems *in vitro* is promising, the potential for large-scale generation of infective oocysts *in vitro* has yet to be realised (Bhalchandra *et al.* 2018).

 To enable the study of a wider range of isolates, we investigated an improved method for oocyst purification, using standard equipment, low-cost reagents, avoiding the addition of any potential chemical contaminants, and removing background biota, thus enabling the investigation of clinical samples without animal propagation. We used updated MALDI-TOF MS techniques to demonstrate proof of principle for the preparation of oocysts from clinical samples, suitable for further investigation by mass spectrometry.

# **Materials and Methods**

## **Samples**

*Cryptosporidium-*positive stools from five diarrhoea patients were selected and anonymised from those submitted for epidemiological surveillance at the CryptosporidiumReference Unit in Swansea (Chalmers *et al.* 2009). Isolates were characterised using a species-specific real-time PCR for *C. parvum* and *C. hominis* targeting the *Lib13* and *A135* genes respectively (Robinson *et al.* 2020), and sequencing part of the *gp60* gene (Alves *et al.* 2003). Stools had been stored refrigerated without preservatives for up to three months at the time of processing. They were selected based on low cycle threshold (Ct) values and characteristic amplification profiles in the real-time PCR, and had an initial volume of at least 15 ml (maximum 30 ml).

 For comparison, highly purified *C. parvum* Iowa oocysts propagated in experimentally infected calves were purchased (Waterborne™, Inc., USA). A *Cryptosporidium*-negative (by RT-PCR and stained microscopy) stool sample and nuclease free water (NFW) (VWR International UK) were processed in parallel with the samples.

## **Oocyst purification**

Oocysts were semi-purified from clinical stools by salt flotation (Ryley *et al.* 1976), re-suspended in 1 ml reverse osmosis (RO) water, and enumerated by duplicate counts using a Neubauer improved disposable haemocytometer (C-Chip™, Digital Bio, Korea). Particularly dirty oocyst suspensions (brown in colour) were mixed 2 : 1 with Tris-EDTA buffer. Further purification was performed by layering the semi-purified oocyst suspension (1 to 1.5 ml) on a potassium bromide (KBr) (Sigma-Aldrich, Germany) gradient and subjecting to ultracentrifugation based on a previously published protocol (Entrala *et al.* 2000). To reduce residues that might impact the spectra produced (Matsubayashi *et al.* 2005), the Entrala protocol was modified by washing the white band of oocysts (1 to 2 ml) thrice by centrifugation at 1000 *g* for 5 min with three times the volume of NFW instead of using phosphate buffer saline. Duplicate aliquots for re-counting oocysts were taken from the pellet before the third and final re-suspension-centrifugation sequence.

 The propagated, purified *C. parvum* Iowa oocyst suspension purchased from Waterborne Inc. was inspected microscopically for any abnormalities and visible contaminants before being washed to remove salts as described above.

 The pellet sizes were estimated and three times the volume of ice-cold 100 % absolute ethanol (EtOH) (VWR International, UK) was added to provide sterile oocyst storage (Jarnuczak *et al.* 2016). The tubes were sealed with film (Parafilm M, VWR International, UK) and transported overnight on dry ice to the Institute of Infection, Veterinary and Ecological Veterinary Sciences, University of Liverpool prior to preparation for MALDI-TOF MS.

## **Sample extraction and preparation for MALDI-TOF MS**

Oocyst suspensions were warmed to room temperature, and the EtOH removed initially by pipette and finally by evaporation until visibly no longer present. Depending on pellet size, 10-50 µl (approximately five times the volume of the pellet) of 70 % aqueous formic acid (Sigma-Aldrich, Germany) was added, mixed by pipetting and left to stand at room temperature for 2 min. An equal volume of 100 % acetonitrile (Honeywell, France) was added, mixed by pipetting and the tubes centrifuged in a fixed head microfuge at 13 000 *g* for 2 min. Eight, 1 µl aliquots of each sample supernatant were spotted onto the target plate (MSP 96 target polished steel BC, Bruker Daltonik GmbH, Germany) and, for calibration purposes, a 1 µl aliquot of bacterial test standard was added to the plate (BTS, Bruker Daltonik GmbH, Germany). The target plate was air-dried at room temperature. Bruker HCCA matrix (α-Cyano-4-hydroxycinnamic acid) (Bruker Daltonik GmbH, Germany) was prepared according manufacturer’s instructions by adding 250 µl organic solvent to a final concentration of 2.5 mg ml-1 HCCA and vigorously mixed until all crystals dissolved. Within 30 min of the complete drying of the aliquot spots, 1 µl of matrix solution was pipetted on top of each spot and allowed to air dry.

**Mass spectrometry and spectral analysis**

The sample extracts where measured using flexControl software on a microflex LT / SH MALDI-TOF mass spectrometer (Bruker DaltoniK GmbH, Bremen, Germany). Spectra were recorded in a linear positive mode at an accelerated voltage of 19 kV in the range from 2 to 20 kDa. For each measurement, 240 individual spectra (40 laser shots at 6 different raster positions) were collected and averaged, and for each of the eight target spots this was performed 3 times. For every analysed sample type, the spectra were first manually inspected and evaluated to ensure that spectra were obtainable with the standard instrument AutoX settings and the standard respective laser settings. In total, for each sample, up to 24 mass spectra were accumulated to create a main spectral projection (MSP). Ideally, at least 20 mass spectra would be required to reach the minimum requirements in Flex Analysis when checking the quality of the peaks, but a less stringent approach was adopted in this exploratory investigation and the available mass spectral data sets were used to create a MSP for each sample. Any flat line or outlier peaks removed initially were further investigated by checking the mass peak shifts were within a tolerance of 500 ppm. External calibration was performed with the BTS (Bruker Daltonik GmbH Bremen, Germany).

 Once the data were analysed and quality controlled, the MSPs were created in the MALDI Biotyper Compass Explorer software 4.1.100 using the “MSP creation standard method”. All steps were performed according to the manufacturer's recommendations. The MSPs where then placed into their own respective library project tree in the Biotyper Software. Each MSP was investigated for any bacterial, fungal or yeast matches against the latest Biotyper v9.0 (8468 entries), Mycobacterial v6.0 and Fungi v3.0 libraries. For taxonomical analyses, the individual *Cryptosporidium* mass spectra as well as the MSPs were used for Biotyper matchings against each other.

# **Results**

Five stool samples from diagnosed cryptosporidiosis patients were selected for oocyst purification, extraction and MALDI-TOF MS preparation, based partially on their low PCR Ct scores (~20-25) and differing species and gp60 subtypes (Table 1). Three samples contained *C. parvum* and two *C. hominis*. Approximate haemocytometer counts after salt flotation indicated >1x106 oocysts ml-1 in each suspension so all five progressed to KBr gradient purification without significant loss of oocysts in most samples (Table 1). For the propagated, purchased *C. parvum* oocyst suspension (Waterborne Inc.) the certificated count was recorded (Table 1).

 Homogenous sample extracts prepared for MALDI-TOF MS from whole oocysts (containing sporozoites) produced MSPs from three of the five candidate clinical stool samples, two *C. parvum* and one *C. hominis* (Table 1). Preparation from stool 1 (1.45 x 107 oocysts) produced 21 successful spectra, stool 2 (5.2 x 105 oocysts) produced 22, stool 3 (3.54 x 106) produced 14 and the purchased *C. parvum* suspension (1 x 107 oocysts) produced 19 spectra (Table 1; Fig. 1). MSPs were not achieved for stools 4 and 5 as insufficient aliquots generated spectra for any detection threshold to be reached; these suspensions had the lowest *Cryptosporidium* biomass prepared for analysis indicated by stool Ct values >25 and oocyst suspension counts < 2 x 105 ml-1 (Table 1). Furthermore, suspension 4 was contaminated with mould spores. Neither the negative control stool (which did not contain *Cryptosporidium* oocysts) nor the NFW regent control produced any mass spectra.

The MSPs produced were distinct and did not map to any other organisms in the Bruker database (data not shown). The MSPs separated well when queried against each other and each sample matched only themselves. Not all spectral markers were present in all samples (Table 2); those that were present in multiple samples displayed different levels of intensity (Figure 1).

#  A spectral marker with a mass to charge (m/Z) ratio of 5830 was apparent in all three *C. parvum* samples but not the *C. hominis* sample (Table 2; Figure 2). There was visible reproducibility across the three lanes for each sample (Figure 2).

# **Discussion**

*Cryptosporidium* is an important cause of gastroenteritis globally, for which preventative measures can be challenging due to its environmental survival, transmissibility, lack of vaccine and limited treatment options. Although there is an increasing abundance of genomic data, peptide and protein data from mass spectrometry are needed to provide experimental evidence for protein predictions (Sanderson *et al.* 2008). Understanding the structure and function of proteins used in cellular invasion and host protein interactions will assist in understanding infection processes (Alberts *et al.* 2002; Sanderson *et al.* 2008), and identifying potential vaccine targets and supporting vaccine design (Manque *et al.* 2011). Furthermore, advances in clinical proteomics are important for the identification of diagnostic biomarkers (Aldred *et al.* 2004) and for informing species- and subtype-specific clinical manifestations (Cama *et al.* 2008).

 We were able to highly purify whole *Cryptosporidium* oocysts (including the sporozoites) from typically low volume human clinical stool samples, prepare extracts from oocyst suspensions while minimising biomass loss, and create MALDI reference entries (in the form of MSPs) for MALDI Biotyper analysis. Production of mass spectrometry data has previously been shown only using experimentally propagated oocysts to varying degrees of success ( Magnuson *et al*. 2000; Glassmeyer *et al.* 2007; Sanderson *et al*. 2008; Lee *et al.* 2011; Matsubayashi *et al.* 2013; Haserick *et al.* 2017), and the large quantity of oocysts required has prevented the use of such methodologies for clinical screening and diagnostic applications. Our data indicated that stools with an initial Ct value of < 25 in our real-time PCR (Robinson *et al.* 2020) produced > 2 x 105 purified oocysts. This Ct value is typically observed in only about five percent of *Cryptosporidium*-positive stools tested at the Cryptosporidium Reference Unit, with the majority of samples in the 25-35 range. Biomass is an important factor in the application of MALDI-TOF, and is likely to have affected the number of spectra available. Even where mass spectra were generated, only half the samples produced the ideal threshold of 20 spectra for Flex Analysis and a less stringent approach was used. This included the experimentally propagated oocysts, indicating that additional optimisation of the extraction method and the MS accumulation is needed. Nevertheless, we have shown that it is possible to produce MSPs from clinical samples as well as experimentally propagated and harvested oocysts. This provides a milestone towards other MS applications to clinical samples. Although, in this proof-of principle study, identification of proteins present in each sample was not performed, these methods will allow for further investigation of *Cryptosporidium* proteomes in a more ethically sustainable way than from limited isolates propagated *in vivo.*

 Our spectra were distinct as they did not map to other organisms in the Bruker database and they separated well from one another when queried (Figure 1). This indicated that spectral interference due to contamination was unlikely and that there were no signs of cross contamination; this was further supported by the flat lines from the negative controls. The distinct and specific MSPs were produced from taxonomically diverse *Cryptosporidium* samples as indicated by their species and gp60 subtype. Some spectral marker peaks were present in only one or two of the samples and were consistently identified in each analyte, whilst other markers occurred in each sample (Table 2). For example, spectral marker 5830 m/z was only found in the *C. parvum* samples, and although more *C. hominis* samples (as well as other species) need to be investigated this may indicate a species-specific marker (Figure 2). However, the full set of peaks for all isolates where not scrutinised in finite detail, and there could well be additional matching peaks (for example, among subtypes) and further work would need to be done to look into this. For this proof of principle study we only focused on the most predominant peaks visible. Those markers that were present in multiple samples displayed different levels of intensity and although this might indicate different abundance, we acknowledge that the sample preparation is not yet optimised. With optimisation, the presence of what might be subtype-specific proteins and recurrent proteins could be profiled and then used as diagnostic biomarkers. Nevertheless, the current protocol allowed us to reliably detect reference spectra for different *Cryptosporidium* spp. from clinical samples. Using higher resolution and tandem mass spectrometry instruments in the future will allow us to further characterise the spectral peaks detected and their functional relevance.

 In this study, sample selection was a rather restrictive process as the majority of clinical samples investigated initially were of a low volume which would not provide sufficient *Cryptosporidium* biomass for analysis after purification which inevitably leads to some oocyst losses. Collecting larger stool volumes from patients or ensuring larger volumes are submitted would be a requisite for further investigations. The modified purification protocol developed in this study was not laborious, used traditional parasitological techniques, and was completed within a working day but with optional stopping points available. Salt flotation, the KBr gradient, and wash solutions were selected for the purification to inflict minimal damage or modification to oocyst surface proteins. Further refining the preparation to separate oocyst shells and sporozoites (the infective stage of the parasite) would facilitate useful analysis of their individual proteomes, but would require an excystation step and separation processes.

 Although the biomass requirements for MALDI-TOF and other mass spectrometry analyses have not been clearly defined, our results show that it can be a challenge to purify sufficient oocysts from some routinely submitted diagnostic stool samples, but the sample preparation measures described above enabled sufficient material to be presented to the MALDI target for three out of five samples. However, other mass spectrometry platforms may provide greater sensitivity. MALDI was selected for this proof-of-principle study because of its availability and recent application in microbial and protein identification (Webster and Oxley 2012; Haslam *et al.* 2016).

 We have demonstrated, using MALDI-TOF MSPs, that it is feasible to generate and compare *C. parvum* and *C. hominis* from clinical samples without propagation. The next steps are to create new MS from different genotypes of these species that can be run against the MSPs that have been created, compare samples of other species, and develop efficient sample preparation and separation of sporozoites from oocyst shells to allow analysis of their proteomes.

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**Conflict of interest**

The MALDI-TOF reagents were provided by Bruker.

**Author contribution**

RG and RMC were responsible for the conception and design of the study, acquisition and analysis of data, drafting and critical review of the manuscript, and approved the final submitted version. ET contributed to the acquisition and analysis of data, drafting and critical review of the manuscript, and approved the final submitted version.

DX, GP-C, GR, DT and FZ contributed to the acquisition and analysis of data, critically revised the manuscript, and approved the final submitted version.

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**Table 1.** *Cryptosporidium spp.* clinical stool samples and experimentally propagated oocyst suspensions prepared for MALDI-TOF mass spectrometry.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample** | **Real-time PCR Ct value** | **Species and** **gp60 subtype** | **Semi-purified oocysts ml-1** **(after salt flotation)** | **Purified oocysts ml-1** | **MALDI-TOF spectra** |
| Clinical stool 1  | 20.54 |  *C. parvum* IIdA19G1 | 1.09 x 107 | 1.45 x 107 | 21 MS; MSP prepared |
| Clinical stool 2  | 24.43 | *C. parvum* IIaA17G1R1 | 2.30 x 106 | 5.20 x 105 | 22 MS; MSP prepared |
| Clinical stool 3  | 24.00 | *C. hominis* IbA10G2 | 4.95 x 106 | 3.54 x 106 | 14 MS; MSP prepared |
| Clinical stool 4  | 25.01 | *C. parvum* IIaA15G2R1 | 1.45 x 106 | 1.88 x 105 | Flat line |
| Clinical stool 5  | 25.62 | *C. hominis* IbA10G2 | 1.76 x 106 | 2.75 x 104 | Flat line |
| Experimentally propagated  | ND | *C. parvum* IIaA17G2R1 | ND | 1.00 x 107  | 19 MS; MSP prepared |

ND = not done; MS = mass spectra, MSP = mass spectral projection

**Table 2.** Examples of unique and shared spectral markers present in *C. parvum* and *C. hominis* samples of different gp60 subtypes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Mass spectra m/z | Clinical stool 1 *C. parvum* IIdA19G1 | Clinical stool 2 *C. parvum* IIaA17G1R1 | Clinical stool 3*C. hominis*IbA10G2 | Experimentally propagated *C. parvum* IIaA17G2R1 |
| 3313 | ✓ |  |  | ✓ |
| 3420 |  | ✓ |  |  |
| 3443 | ✓ | ✓ |  |  |
| 3466 | ✓ |  |  |  |
| 4441 | ✓ |  |  |  |
| 5179 |  |  | ✓ |  |
| 5830 | ✓ | ✓ |  | ✓ |
| 5950 |  |  | ✓ |  |
| 6451 | ✓ |  |  | ✓ |

# **Figure 1.** MALDI-TOF mass spectra of *C. parvum* and *C. hominis* oocysts. Graphs generated using Flex Analysis software.

**Figure 2.** Image of a portion the gel produced by the MALDI Biotyper Compass Explorer showing three representative mass spectra for each sample. Lanes 1-3 Stool 1 *C. parvum*, Lanes 4-6 Stool 2 *C. parvum*, Lanes 7-9 Stool 3 *C. hominis*, Lanes 10-12 Experimentally propagated *C. parvum*.