# *Cryptosporidium parvum*:

# cloning and characterisation of a *superoxide dismutase* gene and

# ozone inactivation studies of oocysts

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## Summary

During this project, a *superoxide dismutase* (SOD) gene was identified from the parasitic protozoa *Cryptosprodium parvum*. The gene was isolated by PCR amplification from genomic DNA with degenerate primers designed according to homologous SOD sequences from related species. Analysis of the partial gene sequence obtained from this and additional library screening approaches suggested that it encoded for an iron-dependant superoxide dismutase protein.

The *SOD* gene was also amplified from a range of Type I and Type II *C.parvum* isolates to investigate sequence conservation between genotypes. Seven polymorphic sites, distinct between the different genotypes, were identified by sequence alignment. This observation suggested that the *SOD* gene could be a candidate molecular marker for genotyping *C.parvum* oocysts, which is of central importance for the control of infection

Previously, variation in susceptibility to ozone exposure has been observed between *C.parvum* oocysts. This aspect was investigated further with genotypically defined oocysts to determine whether this differential susceptibility is dependent on *C.parvum* genotype. Although variation in viability of oocysts was observed under controlled and reproducible conditions, these differences do not appear to be dependent on *C.parvum* genotype.

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## Abbreviations

| | |
|---|---|
| **AIDS** | Acquired immunodeficiency syndrome |
| **cDNA** | Complementary deoxyribonucleic acid |
| **COWP** | *Cryptosporidium* oocyst wall protein |
| **Cu/Zn-SOD** | Copper/Zinc dependent superoxide dismutase |
| **DAPI** | 4'6-diamidino-2-phenylindole |
| **DEPC** | Diethyl pyrocarbonate |
| **DIC** | Differential interference contrast |
| **DIG** | Digoxygenin |
| **DIG-dUTP** | Digoxygenin labelled deoxyuridine triphosphate |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleotide triphosphate |
| **EDTA** | [Ethylenedinitrilo]tetracetic acid |
| **EtOH** | Ethanol |
| **Fe-SOD** | Ferrous dependent superoxide dismutase |
| **FITC** | Fluorecein isothiocyanate |
| **HBSS** | Hanks balanced salt solution |
| **HGMP** | Human Genome Mapping Project |
| **HIV** | Human immunodeficiency virus |
| **$H_2O$** | Hydrogen dioxide |
| **$H_2O_2$** | Hydrogen peroxide |
| **HSP70** | Heat shock protein 70 |
| **KOAc** | Potassium acetate |
| **LB** | Luria broth |

| | |
|---|---|
| **MBSU** | Molecular Biology Support Unit |
| **$MgSO_4$** | Magnesium sulphate |
| **$MgSO_4 \cdot 7H_2O$** | Magnesium sulphate heptahydrate |
| **Mn-SOD** | Manganese dependent superoxide dismutase |
| **mRNA** | Messenger ribonucleic acid |
| **NaCl** | Sodium chloride |
| **NADH** | Nicotinamide adenine dinucleotide |
| **NADPH** | Nicotinamide adenine dinucleotide phosohate |
| **NaOH** | Sodium hydroxide |
| **NBT** | Nitrobluetetrazolium |
| **NBT/BCIP** | Nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl phosophate |
| **NCBI** | National Centre for Biotechnology Information |
| **NZY** | NZ amine (casein hydrolysate) and yeast |
| **$O_2$** | Oxygen |
| **$O_2^-$** | Superoxide radical |
| **$O_3$** | Ozone gas |
| **OD** | Optical density |
| **ORF** | Open reading frame |
| **PAC** | P1 derived artificial chromosome |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **$^{32}$P-dATP** | Radioactivly labelled deoxyadenine triphosphate |
| **PI** | Propidium idodide |
| **RACE** | Rapid amplification of cDNA ends |

| | |
|---|---|
| **RFLP** | Restriction fragment length polymorphism |
| **RNA** | Ribonucleic acid |
| **RNase** | Ribonuclease |
| **rRNA** | Ribosomal ribonucleic acid |
| **RT** | Room temperature |
| **RT-PCR** | Reverse transcriptase polymerase chain reaction |
| **RO** | Reverse osmosis |
| **ROS** | Reactive oxygen species |
| **SDS** | Sodium dodecyl sulphate |
| **SNP** | Single nucleotide polymorphism |
| **SOD** | Superoxide dismutase |
| **SPDL** | Scottish Parasite Diagnostic Laboratory |
| **SSCP** | Single-strand conformation polymorphism |
| **TRAP C1** | Thrombospondin related anonymous protein C1 |
| **TRAP C2** | Thrombospondin related anonymous protein C2 |
| **Tris** | Tris[hydroxymethyl]aminomethane |
| **Tris-HCl** | Tris hydrochloride |
| **µCi** | micro Curie |
| **UV** | Ultra violet |
| **X-gal** | 5-bromo-4-chloro-3-indol-D-galactopyranoside |

## Declaration

I declare that the work recorded in this thesis is entirely my own, unless otherwise stated and that it is of my own composition. No part of this work has been submitted for any other degree.

Judith Gallagher B.Sc

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# Chapter One: Introduction

## 1.1 *Cryptosporidium:* history and biology

*Cryptosporidium* was first identified in laboratory mice in 1907 (Tyzzer, 1907). The full extent of the importance of the parasite in relation to outbreaks of acute diarrhoea in a range of hosts was not realised until half a century later (Salvin, 1955). However, few cases of human cryptosporidiosis had been recorded previous to the emergence of acquired immunodeficiency syndrome (AIDS) in the early 1980's, where a correlation between the two infections highlighted the direct significance of this pathogen to humans (Crawford & Vermund, 1988). Since then, *Cryptosporidium parvum*, the sole species considered infectious to humans, has been investigated extensively.

To date, up to ten valid species of *Cryptosporidium* have been identified (Fayer, *et al*, 2000), although many more species have been proposed (O'Donoghue, 1995, Morgan, *et al*, 2000 (b)). The most commonly encountered species are *C.parvum* in various mammalian hosts, *C.wrairi* in guinea pigs, *C.muris* in mice, *C.felis* in cats, *C.baileyi* and *C.meleagridis* in birds, *C.serpentis* in reptiles and *C.nasorum* in fish. With the exception of the larger oocysts of *C.muris*, the different *Cryptosporidium* species are morphologically very similar. Molecular differentiation has identified two distinct *C.parvum* genotypes which are classified as Type I isolates, that are infectious to humans only, and Type II isolates, that are able to infect both humans and animals (Peng, *et al*, 1997, Spano, *et al*,

1997). An apparent lack of genetic recombination between the two *C.parvum* genotypes has also raised debate on the classification of *C.parvum* isolates as a single species.

*Cryptosporidium parvum* has been identified as the causative agent of infection in over 150 mammalian species, yet, debate ensues in relation to the classification of isolates within this species (Morgan, *et al*, 1999(a), Morgan, *et al*, 1999(b), Fayer *at al,* 2000). Isolates are classified according to host specificity, oocyst morphology and site of infection and inconsistencies within *C.parvum* have aroused debate about the credibility of a single species. Indeed, the number of valid *Cryptosporidium* species recognised by different researchers within the field also varies, highlighting the necessity for a universal speciation and classification system, for which further investigation of the parasite is required.

## 1.2 Epidemiology

*Cryptosporidium parvum* is a protozoan parasite, belonging to the phylum Apicomplexa. It infects the microvillus border of the gastrointestinal epithelium of the host following ingestion of oocysts (Crawford & Vermund, 1988, O'Donoghue, 1995, Tzipori & Griffiths, 1998). Infection results in self-limiting, but often severe, diarrhoea in immunocompetent individuals. However, chronic infection may occur in immunocompromised patients, including those suffering from immunodeficiency diseases or receiving immunosuppresive therapy, and can be fatal under such circumstances (Xiao *et al,* 2000, Crawford & Vermund, 1988, Griffiths, 1998). The

impact of the opportunistic nature of *C.parvum* on human immunodeficiency virus (HIV) and AIDS patients is well documented (Kasper, & Buzoni-Gatel, 1998, Byeveld, *et al*, 1999, Treitinger, *et al*, 2000, Morgan, *et al*, 2000(a)). Although present in both developing and developed countries (Crawford & Vermund, 1988), factors such as poor sanitation and malnutrition in young children are linked with an increased incidence of cryptosporidiosis. Others at increased risk include those in contact with infected individuals or animals, young children and travellers to endemic areas (Crawford & Vermund, 1988).

Transmission of *C.parvum* occurs from an infected host to a susceptible individual by the faecal-oral path. There are a number of possible routes of infection including person to person, animal to person, waterborne or foodborne contamination and possibly airborne transmission, although evidence for the latter is lacking (Griffiths, 1998). Water contamination has been reported as the source of a number of cryptosporidiosis outbreaks throughout the world (Smith & Rose, 1998, Fricker & Crabb, 1998). *C.parvum* oocysts, the infective form of the pathogen, enter the water system from agricultural sources and sewage output and have been found to persist in public water supplies following the current filtering and purification process (Smith & Rose, 1998, Fayer *et al,* 2000, Crawford & Vermund, 1988, Griffiths, 1998, Fricker & Crabb, 1998). Further investigation of the robust nature of *C.parvum* is required to develop a reliable method for removal or inactivation of oocysts to limit the threat of waterborne cryptosporidiosis.

## 1.3 Life cycle

The monoxenous life cycle of *C.parvum* requires ingestion of thick-walled oocysts by the host. Each oocyst contains four sporozoites that are released into the gut and attach, by means of a single apical complex, to the epithelial cells of the intestine. Infection is limited to the jejunum and ileum of immunocompetent humans but has been reported throughout the gastrointestinal tract of immunocompromised hosts (Crawford & Vermund, 1988). The sporozoites penetrate the epithelium and form type I meronts, which, in turn, produce four type II merozoites. This asexual stage is then followed by an obligatory sexual stage of development with fertilisation between the micro and macrogametes, both of which are produced by type II merozoites. The resulting diploid zygote forms a protective oocyst wall. The majority thick-walled oocysts are excreted by the host and may spread infection. Thin-walled oocysts, however, are able to sporulate within the host, producing four haploid, infectious sporozoites, thus enabling autoinfection (O'Donoghue, 1995).

## 1.4 Water purification

Many outbreaks of cryptosporidiosis have been traced back to drinking water supplies, with swimming pools, rivers, lakes and ground water being implicated as sources of contamination (Smith & Rose, 1998, Fricker & Crabb, 1998). In most cases, the number of people affected have been limited to hundreds of individuals, although, on occasion, larger outbreaks have affected hundreds of thousands of individuals; for example, in an

outbreak in Milwaukee, Wisconsin in 1993, more than 400,000 individuals were infected (MacKenzie, *et al*, 1994).

As waterborne transmission is accountable for major outbreaks of cryptosporidiosis, the need for an effective water purification and disinfection technique is essential. This requirement is highlighted by the fact that no effective drug therapy has been identified for cryptosporidiosis (Tzipori, 1998). Therefore, prevention by control of transmission of the parasite has important implications. Present methods applied for water purification involve coagulation, sedimentation and filtration to remove contaminants from the water supplies. This process is highly effective in removing the majority of *C.parvum* oocysts. However, viable oocysts can pass through the purification process and persist in drinking and recreational water supplies.

A range of disinfectants have been considered in an attempt to identify the most effective method for *C.parvum* inactivation (Campbell, *et al,* 1982, Korich, *et al.,* 1990, Rennecker, *et al.*, 1999). Korich *et al.* (1990) included chlorine, chlorine dioxide, monochloramine and ozone in their investigation. However, they suggest that, with the possible exception of ozone, inactivation of *C.parvum* oocysts would not be achieved with any single disinfectant. The robust nature of the oocyst renders many disinfectants inadequate for inactivation of *C.parvum* oocysts and the occurrence of swimming pool-related outbreaks highlights the relative ineffectiveness of chlorine as a single disinfectant. Further investigation to assess potential disinfectants is required.

### 1.5 Ozone and superoxide dismutase

Ozone is produced with other reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$) and the superoxide radical ($O_2^-$), as a consequence of the sequential reduction of oxygen to water during aerobic metabolism. High levels of these oxidants are disruptive to the cell membrane, nucleic acids and proteins (McGonigle, *et al*, 1998). Defence against such cellular damage has evolved, resulting in antioxidant enzymes including superoxide dismutase (SOD), catalase and the glutathione system.

SOD is present in a vast range of organisms and catalyses the dismutation of superoxide radicals to produce oxygen and hydrogen peroxide, as shown in the following equation (McCord & Fridovich, 1969):

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

By scavenging the $O_2^-$ radical, superoxide dismutase plays a role in neutralising the toxicity of reactive $O_2$ intermediates within the cell. It is also possible that SOD plays a role in protecting against the host immune response following infection. The production of oxygen radicals is known to be associated with the activity of macrophages and other cells involved in the immune response. The role of a cell-mediated immune response is well established in other intracellular protozoan parasites (Nathan, *et al,* 1979; Michalski & Prowse, 1981; Murray, 1981, 1983; Britten, & Hughes, 1986; Sibley, *et al*, 1986).

However, there have been no studies to investigate how *C.parvum* deals with any free radicals that might be produced during the host immune response.

The superoxide dismutase enzyme was first described by McCord and Fridovich in 1968 when they presented evidence of enzymatic activity which catalysed the above dismutation reaction and associated the activity with a previously described copper-containing protein (McCord & Fridovich, 1968, McCord & Fridovich, 1969). Further investigation revealed this enzyme to be dependent on both copper and zinc (Fridovich, 1975). Two further types of SOD were later confirmed, differentiated by the metal co-factor they are dependent on: manganese-dependent (Mn-SOD) and iron-dependent (Fe-SOD). More recent studies have established methods for distinguishing between the different types of SOD and these include molecular characterisation and differential susceptibility assays (Baert, *et al*, 1999(a), Bécuwe, *et al*, 1996). Fe-SODs and Mn-SODs are considered to have evolved from a common ancestry due to their closely related primary and tertiary structures (Smith & Doolittle, 1992), whereas Cu/Zn-SOD is likely to have evolved along a different evolutionary route (Tainer, *et al*, 1982). Iron-dependent superoxide dismutase has been isolated from a number of other protozoan parasites (Tannich, *et al*, 1991, Bécuwe, *et al*, 1996, Viscogliosi, *et al*, 1998, Baert, *et al*, 1999(a), Baert, *et al*, 1999(b), Odberg-Ferragut, *et al*, 2000) and appears to be the dominant SOD present in such organisms.

## 1.6 Genetic variation within *Cryptosporidium parvum*

Two distinct genotypes have been identified within the *Cryptosporidium parvum* species and are characterised by host specificity of the isolate: Type I oocysts infect humans only, while Type II oocysts infect both humans and animals. Further analysis of the *C.parvum* genome has uncovered a number of loci that show polymorphism between *C.parvum* genotypes. Identification of such polymorphic loci will contribute to the development of an accurate genotyping system, which is essential in understanding the genetic structure of *C.parvum* populations and would facilitate in establishing the routes of transmission of the parasite. This would aid in the control of *C.parvum*, which is of prime importance as there is no effective treatment for infection.

The genetic variation within *C.parvum* was initially considered by means of PCR-based methods including RFLP and more recently, microsatellite analysis (Aiello, *et al*, 1999, Feng, *et al*, 2000, Caccio, *et al*, 2000) and single-strand conformation polymorphism (SSCP), (Gasser, *et al*, 2001). The necessity for a PCR-based method stems from the lack of a suitable technique for *in vitro* cultivation of *C.parvum* and, therefore, limited availability of oocysts. Such molecular methods also allow for rapid and accurate analysis of isolates, which would be of central importance for control of an outbreak of infection.

Initial investigations of the polymorphic nature of the *C.parvum* genome utilised the restriction fragment length polymorphism (RFLP) method of a small number of known

polymorphic genes. More recently, identification of simple tandem repeat sequences, or microsatellite markers, has proved to be a source of highly polymorphic sequences and have been used for high-resolution genotyping and genome mapping projects of other organisms. Such repetitive regions can be identified from genome sequencing databases and are useful for distinguishing between genotypes with very subtle length polymorphisms.

The attention given to microsatellite markers may change in favour of the identification of specific sequences within the genome containing single nucleotide polymorphisms (SNPs). The stability of SNPs is favoured over the high mutation rate typical of repetitive sequences including microsatellite sequences. Indeed, the very feature that allows microsatellite sequences to display rich polymorphism also suggests that they are potentially unstable molecular markers. However, the stability of microsatellite markers for *C.parvum* remains to be examined. Nonetheless, they are likely to prove valuable tools for determining the population structures of *C.parvum*.

The opportunity for development of a molecular genotyping tool will be greatly increased with further identification of polymorphic markers from *C.parvum*. Preliminary research has identified several candidate sequences (Spano, *et al*, 1998) and also with the β-Tubulin gene (Rochelle, *et al*, 1999, Sulaiman, *et al*, 1999 & Caccio, *et al*, 1999), the thrombospondin-related anonymous protein 2 of *C.parvum* (TRAP C2) gene (Sulaiman, *et al*, 1998) and the heat shock protein 70 (HSP70) and small subunit (SSU) of ribosomal RNA genes (Gasser, *et al*, 2001). Spano *et al* (1997) also found that a simple *Rsa*I

endonuclease digest could distinguish between the polymorphic PCR-RFLP products. Development of a genotyping system encompassing these results and further polymorphic sequences would allow for rapid and specific differentiation between *C.parvum* genotypes.

## 1.7 Project outline

Pervious studies have shown *C.parvum* oocysts to display differential susceptibility to ozone when employed as a disinfectant for water purification (Parker, 1993). Although the biochemical basis of the susceptibility of *C.parvum* oocysts to ozone is not understood, it is possible that superoxide dismutase may play an important role in the antioxidant defence mechanism of the oocysts. Although SOD enzyme activity had previously been reported in *C.parvum* (Parker, 1993, Parker, *et al*, 1993, Entrala, *et al*, 1997), the corresponding gene sequence had not been identified. Therefore, this project aimed to clone and characterise a *superoxide dismutase* gene from *Cryptosporidium parvum* and determine the polymorphic nature of the gene in a range of parasite isolates.

The opportunity arose for a short study on ozone exposure on a panel of *C.parvum* oocysts to investigate any difference in susceptibility between Type I and Type II isolates. Although previous studies had shown differential susceptibility to ozone, no molecular typing system was available at the time and, therefore, no correlation between genotype and susceptibility could be made.

The first phase of this project involved the identification of putative *superoxide dismutase* gene homologues from *C.parvum* genomic DNA. Any candidate sequences were then analysed to characterise further the gene. Polymorphisms within the gene were then identified by cloning and sequencing the *SOD* gene fragment from a number of *C.parvum* isolates, including both Type I and Type II genotypes. Further to these molecular studies, the differential susceptibility of *C.parvum* isolates to exposure to ozone was also investigated. This part of the project was performed in collaboration with the Scottish Parasite Diagnostic Laboratory (SPDL) and with assistance from Ozotech Ltd.

# Chapter Two: Cloning of a *Superoxide Dismutase* gene from *C.parvum*

## 2.1 Introduction

*Cryptosporidium parvum* can be found in the food and water supplies of communities, not only in developing countries, but also throughout the developed world where infection can prove fatal for the young, the old and the immunocompromised. The robust nature of the oocyst, the infective form of the parasite, demands high efficiency from water purification methods and *C.parvum* has shown to survive treatment of current chemical disinfection methods including chlorination. For this purpose, ozonation has been introduced in an endeavour to inactivate completely *C.parvum* oocysts within some public water supplies.

Superoxide dismutase is an antioxidant enzyme that helps to protect against oxidative damage to the organism caused by free radicals, such as ozone (McCord & Fridovich, 1969). SOD is involved in a complex biochemical pathway, which neutralises the damaging affects of reactive oxygen species (ROS) and works in concert with other antioxidants such as catalase and the glutathione system. Previous investigations of the antioxidant enzymes of *C.parvum* (Entrala, *et al.,* 1997) reported that superoxide dismutase activity could be detected within the organism. This lead us to attempt to clone and characterise the superoxide dismutase gene of *C.parvum.*

The anitoxidant defence mechanisms of certain protozoan parasites have been well esablished (Mehlotra, 1996). Entrala *et al.* (1997) reported on the antioxidant enzymes present in *C.parvum.* They performed assays to detect catalase, superoxide dismutase,

glutathione reductase, glutathione peroxidase, glutathione S-transferase, trypanothione reductase and peroxidase activity. They failed to detect all antioxidants considered, with the exception of SOD, which was found to be present at relatively low levels. SOD activity was detected by three methods: (1) generation of SOD by photoactivation of riboflavin and detection by the reduction of nitrobluetetrazolium (NBT) to an insoluble purple formazan (Beyer, & Fridovich, 1987), (2) generation of SOD from molecular oxygen in the presence of manganous ions and detection by the reduction of NAD(P)H (Paoleti, & Mocali, 1990), (3) by using a gel assay. In this final assay, SOD activity was detected as with the NBT assay, but the gels were also incubated with potassium cyanide and $H_2O_2$ to distinguish between Cu/Zn and Mg or Fe dependent SOD. All three assays confirmed the presence of SOD and indicated that the SOD was specifically Fe-dependent. The activity of SOD varied between each assay, from as little as 5.7±1.4 units/mg protein to 53.2±4.2 units/mg protein. As a result, Entrala *et al* (1997) suggested that the failure to detect all other antioxidants considered was due to the absence of mitochondria in *C.parvum* and, therefore, the absence of a classical respiratory transport chain.

Molecular studies of *C.parvum* have increased in recent years, with the HAPPY mapping (Piper, *et al.*, 1998(a), www.mrc-lmb.cam.ac.uk/happy/CRYPTO/crypto-genome.html), expressed sequence tag (EST) (www.ebi.ac.uk/parasites/cparvEST.html) and genomic sequence tag (GST) (Liu, *et al.*, 1999, www.ebi.ac.uk/parasites/cparvGST.html) genome sequencing projects underway. The *C.parvum* genome is thought to be approximately 9.4Mbp in size, consisting of eight chromosomes ranging in size from 0.94Mbp to 1.44Mbp (Caccio, *et al.* 1998, Spano & Crisanti, 2000). Studies have also suggested that the *C.parvum* genome is 60-70% A/T rich (Liu, *et al.*, 1999, Spano & Crisanti, 2000) and

there is a general lack of introns, with only one characterised gene containing a small intron of 85bp (Caccio, *et al.*, 1997).

The initial aim of this project was to identify any *superoxide dismutase* homologous genes from *C.parvum* genomic DNA by means of PCR amplification utilizing *SOD* sequence information from related protozoan parasites. Sequence analysis was used to determine the form of superoxide dismutase present: Fe-dependent, Mn-dependent or Cu/Zn-dependent. Gene expression was investigated by screening a *C.parvum* cDNA library for the *SOD* gene sequence and confirmed by RT-PCR.

## 2.2 Materials and Methods

### 2.2.1 DNA Isolation from *C.parvum* Oocysts

The initial step in the isolation of DNA from *C.parvum* oocysts involved breaking open the robust oocyst wall to release the contents. This was achieved by incubating approximately $1x10^6$ oocysts (suspended in 20 µl $H_2O$) in 200µl of lysis buffer (50mM Tris-HCl (pH8.5), 1mM EDTA, 0.5% SDS). The lysis involved 15 cycles of freezing the oocysts in liquid nitrogen for 1min, followed by thawing at 65°C for 1min. The lysed oocysts were then incubated for >3h at 55°C with 2µl of Proteinase K (10mg/ml, Sigma). The proteinase was inactivated by incubation at 95°C for 20min. The cell debris was pelleted by centrifugation at 20000xg for 5min. The supernatant containing the DNA was removed and the DNA was isolated by phenol/chloroform extraction and ethanol precipitation (Sambrook, *et al*, 1989) and resuspended in 200µl of $H_2O$.

### 2.2.2 PCR Amplification of *SOD* from Type II Genomic DNA

An internal fragment of the *SOD* gene was obtained by PCR amplification from *C.parvum* Type II genomic DNA. Degenerate primers were designed according to Bécuwe *et al* (1996), which describes the identification of a FeSOD gene from *Plasmodium falciparum* (accession number Z49819). An alignment of *SOD* sequences from a number of different organisms, including *Photobacterium leiognathi, Pseudomonas ovalis, Arabidopsis thaliana* and *Entamoeba histolytica* (Bécuwe, *et al,* 1996) highlighted regions of significant homology. Primer design began with amino acid sequences 5' N-F-H-Y-N-K-H 3' and 5'W-E-H-A-Y-Y 3' from the *Plasmodium falciparum* sequence. Positions of variation were identified from the *Toxoplasma gondii* FeSOD sequence (accession number AF029915), which was considered to be more closely related to the *C.parvum*. This highlighted three variable positions within the sense primer sequence. The antisense primer amino acid sequence is 100% conserved throughout all the species included in the alignment. However, as certain amino acids can be encoded for by more than one codon, the most suitable nucleotide sequence for amplification from the *C.parvum* genome was determined by considering the codon usage, dinucleotide preference and the number of simple trinucleotide repeats of this species genome (Liu, *et al*, 1999). This led to the design of degenerate primers FCRP-SOD and RCRP-SOD, shown in table 2.1 and figure 2.1.

The PCR reaction mix comprised of template DNA, dNTP mix at 10mM concentration and 2.5 Units of Taq polymerase (Promega), 1x reaction buffer and magnesium chloride (25mM). The template DNA was initially denatured at 94°C for 5min, followed by 32 cycles of 50s denaturation at 94°C, 50s annealing at 40°C or 50°C and 1min of elongation at 70°C with a final elongation period of 20min at 70°C. The PCR products were analysed by

gel electrophoresis on a 1% agarose gel, stained with ethidium bromide to a final concentration of 0.1% and viewed by UV illumination.

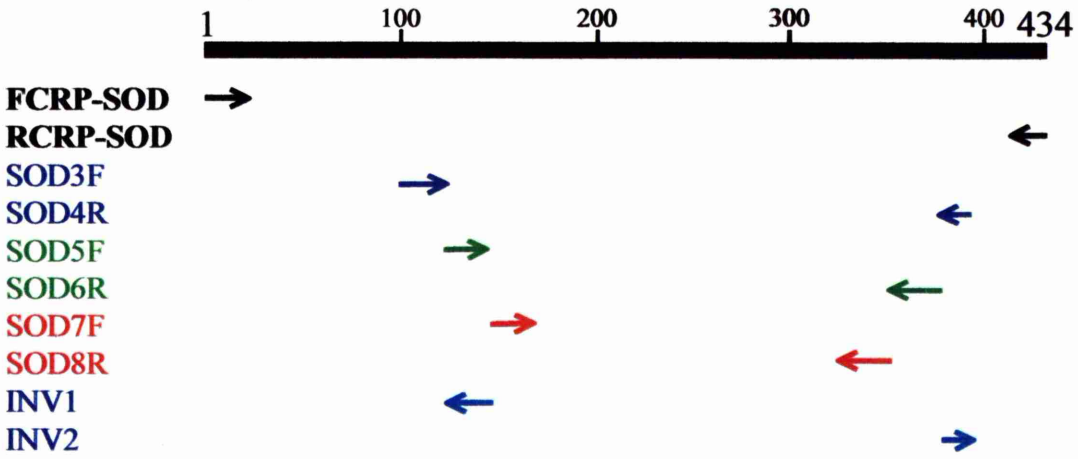



**Figure 2.1 PCR primer positions within the *Cryptosporidium parvum SOD* gene.** All *SOD* specific primers employed in this study are represented here in relation to their position within the gene. All numbers are relative to the internal fragment of the gene, which was isolated by PCR and cloned during this study (Section 2.2). The arrowheads indicate the orientation of the primer and the oligonucleotide primer sequences are shown in table 2.2.

**(a)** **Table 2.1**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (1) Amino acid sequence of upstream *P.falciparum SOD* primer (5'-3') | N | F | H | Y | N | K | H |
| (2) Corresponding *T. gondii* amino acid sequence | Q* | F | H | H* | G* | K | H |
| (3) *T.gondii* nucleotide sequence | CAG | TTT C | CAT C | CAT C | GG $^{T}_{C}$ $^{A}_{G}$ | AAA G | CAT C |
| (4) FCRP-SOD (5'-3') | CAG | TTC | CAT | CAT | GGC | AAG | CAT |

**(b)**

| | | | | | | |
|---|---|---|---|---|---|---|
| (1) Amino acid sequence of downstream *P.falciparum SOD* primer (5'-3') | W | E | H | A | Y | Y |
| (2) Corresponding *T. gondii* amino acid sequence | W | E | H | A | Y | Y |
| (3) *T.gondii* nucleotide sequence | TGG | GAA G | CAT C | GC $^{T}_{C}$ $^{A}_{G}$ | TAT C | TAT C |
| (4) RCRP-SOD (3'-5') | ACC | CTC | GTA | CGA | ATA | ATA |

*- Amino acids that differ between the *P.falciparum* and *T.gondii* sequences

**Table 2.1 Factors considered in the design of the degenerate primers for amplification of the *SOD* gene from *C.parvum* genomic DNA.** (a) The design of the degenerate forward primer involved comparing the amino acid sequences of a highly conserved region of the *superoxide dismutase* gene from (1) *P.falciparum* and (2) *T.gondii*. (3) The *T.gondii* nucleotide sequence was determined and each possible wobble at the third position of the codon was considered. (4) The final primer sequence was designed according to the codon usage and nucleotide preference of *C.parvum* (Liu, *et al,* 1999). (b) The design of the complementary degenerate primer sequence was conducted in the same manner as for the forward primer.

**Table 2.2**

| PRIMER NAME | PRIMER SEQUENCE (5' TO 3') | LENGTH (bp) | GC CONTENT (%) |
|---|---|---|---|
| FCRP-SOD | CAG TTC CAT CAT GGC AAG CAT | 21 | 48 |
| RCRP-SOD | ATA ATA AGC ATG CTC CCA | 18 | 39 |
| SOD3F | TGA AGA GTT CTG GCC CAA TC | 20 | 50 |
| SOD4R | TCC AAG TCC GAG CTT CTC AG | 20 | 55 |
| SOD5F | CTA CAA CAA TGC ATC CCA GAT TTG GAA T | 28 | 39 |
| SOD6R | AGG ATT GTC ACC ATC ATG AGT TTG GAC | 27 | 44 |
| SOD7F | GCA TCA TGC AGG ATA TGT TAC | 21 | 43 |
| SOD8R | TAC ATC ACA GGT TAG AAC TGG | 21 | 43 |
| CYP1F | ACA GTC ATA AGT GCG GCG ACG ATA GT | 26 | 50 |
| CYP2R | TTG CGA TCT GCC GTT TCG ATC CTC C | 25 | 56 |
| INV1 | ATT CCA AAT CTG GGA TGC ATT GTT GTA G | 28 | 39 |
| INV2 | CTG AGAAGC TCG GAC TTG GA | 20 | 55 |

**Table 2.2 Primers used for the cloning of the *C.parvum SOD* gene sequence** The sequence, length and GC content of all the oligonucleotide primers employed in this study. All primer sequences were designed to give 100% homology to the *SOD* sequence, with the exception of FCRP-SOD and RCRP-SOD which were degenerate primers based on the *Plasmodium falciparum* FeSOD sequence (Section 2.2.2 and Table 2.1)

### 2.2.3.1 Cloning of *SOD* Fragment

The 434bp PCR fragment of the putative *SOD* gene was cloned using the TOPO® TA Cloning Kit, with pCR®2.1-TOPO® vector and TOP10F competent cells, following the manufacturer's protocol (Invitrogen). The transformed cells were grown on LB plates supplemented with 50μg/ml ampicillin for selection and 40μl of 40mg/ml X-gal to facilitate blue/white colour selection. Positive colonies were selected and grown overnight in 5ml LB broth supplemented with ampicillin (50μg/ml). Following the preparation of the plasmid DNA with QIAprep Spin Miniprep Kit (QIAGEN), the presence of the PCR fragment insert was confirmed by exonuclease restriction digest with *Eco*RI. Two samples were selected and sequenced in both directions by The Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK.

### 2.2.3.2 Sequence Analysis

Sequencing results were analysed using software from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). All possible open reading frames were considered with the ORF (Open Reading Frame) Finder and homologous sequences were identified by searching the GenBank public database with the BLAST program, also available at NCBI. Sequence alignments were produced using either the ClustalW program available at the European Bioinformatics Institute (www.ebi.ac.uk/) or GeneDoc (www.psc.edu/biomed/genedoc).

### 2.2.4 Southern Hybridisation of *C.parvum* Genomic DNA

*C.parvum* genomic DNA from a Type II isolate was used for Southern analysis of the genome. Four exonuclease restriction enzymes were selected according to the frequency of their recognition site within the known *C.parvum SOD* gene sequence; *Eco*RI and *Nsi*I which both cut once within the known *SOD* sequence and *Sac*I and *Xho*I which do not cut within the known *SOD* sequence. Genomic DNA (1μg) and 20 units of enzyme were used in each overnight digestion. The fragments were analysed by electrophoresis on a 0.8% agarose gel and visualised under UV illumination. The Southern blot was performed as directed by Sambrook *et al* (1989). The DNA was transferred to a Hybond-$N^+$ membrane for hybridization (Amersham Pharmacia Biotech) and cross-linked to the membrane by UV illumination. The membrane was pre-hybridised at 65°C for 2h in hybridization solution (4X SSC, 0.5% SDS, 5x Dernhardt's solution, 40μg/ml denatured salmon sperm and $H_2O$). The probe was prepared by PCR from the *SOD* gene clone (Section 2.2.3.1) and 30ng of the product was labelled with 30μCi of $^{32}$P-dATP according to Prime-It II protocol (Stratagene). The labelled probe was added to the hybridisation solution and hybridisation continued at 65°C overnight.

Following hybridisation, the membrane was washed initially with 4x SSC, 0.1% SDS at 65°C and then with 0.1x SSC, 0.1% SDS at RT. The membrane was then sealed in cling film and exposed to photographic film.

### 2.2.5.1 Screening of P1-derived Artificial Chromosome (PAC) Genomic Filter

A *C.parvum* genomic PAC library, available commercially from Human Genome Mapping Project (HGMP), Cambridge, UK, was selected to obtain the remaining 5' and 3' sequences of the *SOD* gene. The library, consisting of 1602 clones with an average insert size of 38kb and was available as a high-density gridded filter, with each clone spotted twice to give a total of 3204 spots on each membrane. Interpretation of the filter, as indicated by the manufacturers, allows for the identification of a single clone from the library. The library provides 6-fold coverage of the genome.

The filter was screened by Southern hybridisation with a *SOD* specific digoxygenin (DIG) labelled probe. The probe was prepared by PCR amplification with the *SOD* clone (Section 2.2.3.1) as the template, *SOD* gene specific primers: SOD3F and SOD4R (Figure 2.1 and Table 2.2), and DIG-dUTP (Roche UK Ltd). Hybridisation of the filter involved prehybridisation for 30min at 68°C in Rapid-hyb buffer (Amersham Pharmacia Biotech), followed by 3h hybridisation with the DIG-labelled probe at 68°C. The filter was then washed twice with 2X SSC, 0.1% SDS at room temperature, followed by two washes with 0.1X SSC, 0.1% SDS at 68°C. The detection of the digoxigenin was performed with Anti-Digoxigenin-AP Fab fragments and NBT/BCIP (Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) Ready-to-Use Tablets for Colourimetric Detection System (Roche UK Ltd).

### 2.2.5.2 Preparation and Analysis of PAC Clone DNA

The recommended protocol for the isolation of the high molecular weight PAC clones was provided by HGMP. This method involved resuspending the bacterial pellet in 300µl of solution 1 (15mM Tris, pH 8, 10mM EDTA, 100µg/ml Rnase A) and cell lysis by adding 300µl of solution 2 (0.2N NaOH, 1% SDS). Following 5min at RT, 300µl of solution 3 (3M KOAc, pH 5.5) was added and placed on ice for 5min to form a precipitate of protein and bacterial DNA. The precipitate was removed by centrifugation at 12000xg, 4°C, for 10min. The supernatant was added to 800µl of ice-cold isopropanol and placed on ice for a further 5min. The PAC DNA was pelleted by centrifugation at 12000xg, at 4°C, for 15min. The supernatant was discarded and salt was removed by washing twice with 500µl of 70% EtOH and centrifuging at 12000xg for 10 minutes. The DNA pellet was then allowed to air dry before being resuspended in 30µl $H_2O$.

### 2.2.5.3 Subcloning of PAC Clones

PAC clone DNA (~500ng) was digested to completion with three different restriction enzymes: *Eco*RI, *Bam*HI and *Xho*I. The total digest volume was loaded onto an agarose gel and the DNA fragments were separated and transferred to Hybond-$N^+$ (Amersham Pharmacia Biotech). Southern hybridisation of the transferred DNA with a DIG-labelled *SOD* specific probe proceeded, as described in section 2.2.5.1, to identify fragments of DNA containing the *SOD* gene and allow subcloning of the PAC clone insert.

### 2.2.5.4 PCR Amplification of 10-H11 with *SOD* Specific and Vector Specific Primers

Sequence information for the pCYPAC2 vector used in the construction of the PAC library, available at http://bacpac.med.buffalo.edu, was used to design vector specific primers; CYP1F and CYP2R (Table 2.2), specific to the region flanking the insert. Nested *SOD* specific primers were also designed: SOD5F and SOD6R (Table 2.2). PCR amplification with the *SOD* specific and vector specific primers was implemented with the 10-H11 template under standard PCR conditions and also as indicated in section 2.2.5.5.

### 2.2.5.5 Long Template PCR

PCR amplification of the full-length insert from PAC clone 10-H11 and also from the *SOD* gene to the vector sequence was attempted with the Expand™ Long Template PCR System (Roche UK Ltd). The parameters of the PCR reaction were optimised according to the manufacturer's recommendations. A variety of factors were altered in an attempt to identify optimal conditions, including template DNA concentration, magnesium chloride concentration and annealing temperature.

### 2.2.5.6 Inverse PCR

Inverse PCR was used to amplify from the known *SOD* sequence to the flanking regions within PAC clone 10-H11. An overview of this method is shown in figure 2.2. The initial step involved digestion of approximately 1μg of DNA with restriction enzyme *Dpn*II. This enzyme was selected to provide a four-base overhang, to facilitate ligation, and as an enzyme with no recognition sites within the known *SOD* gene sequence. The digested DNA was recovered by phenol/chloroform extraction and ethanol precipitation (Sambrook, *et al*, 1989). The DNA was diluted to give a concentration of 1-10μg/ml and the *Dpn*II fragments were circularized by overnight incubation at 14°C with T4 DNA Ligase (Promega). The circularised DNA was recovered as before and digested with restriction enzyme *Bsa*I. This second restriction enzyme was selected to cut within the known *SOD* sequence and create a linear 'inverted' sequence. PCR amplification of the linear product was performed with primers INV1 and INV2 (Table 2.2 and Figure 2.1). A gradient of annealing temperatures and a range of template concentrations were tested to identify optimal amplification conditions.

**Figure 2.2**

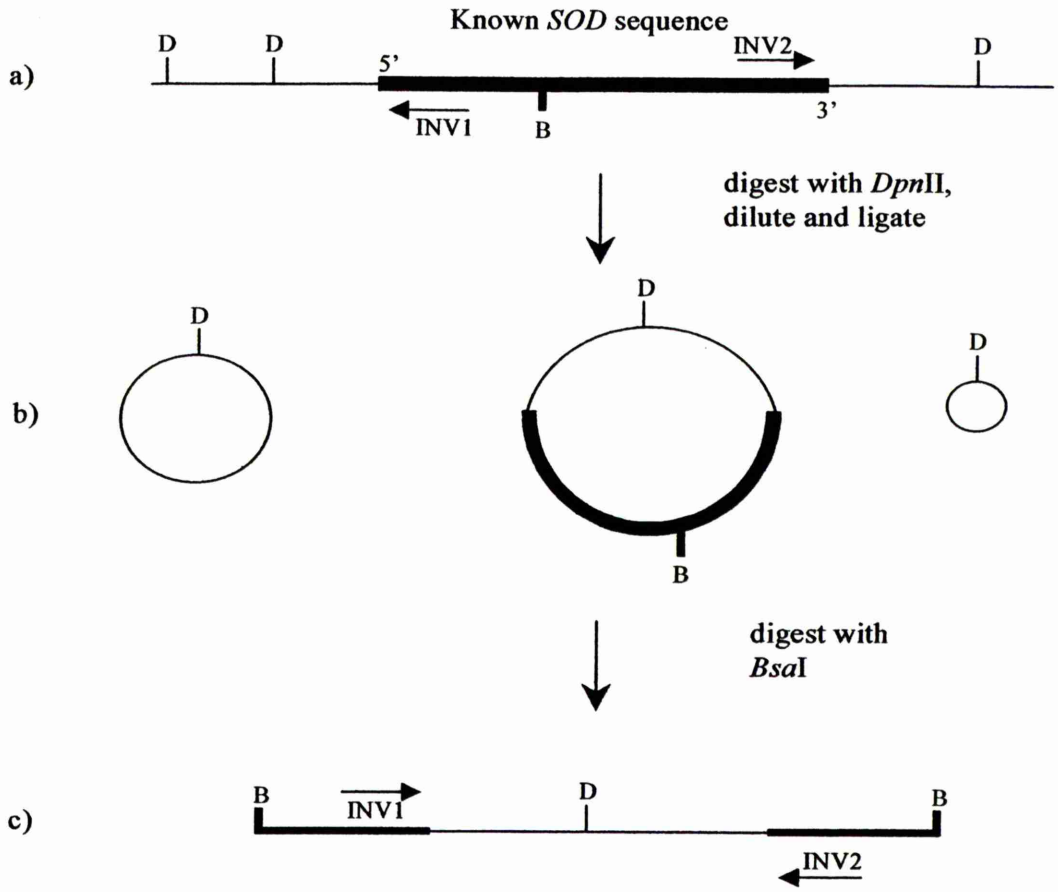



**Figure 2.2 Inverse PCR** The principle of inverse PCR involves the construction of a template for PCR amplification from a known DNA sequence to the flanking region of unknown sequence. a) This is achieved by digesting the sample DNA with a restriction enzyme which does not cut within the known sequence. b) The DNA is then diluted and ligated to form circular fragments of DNA. c) A second restriction enzyme, with a recognition site within the known sequence, is then used to linearise the DNA. This provides a linear template for PCR with specific primers.

### 2.2.6 PCR Amplification of *SOD* from cDNA λZAP Library

The plasmid DNA was purified from the phage vector by standard phenol/chloroform extraction (Sambrook, *et al*, 1989). PCR amplification of the *SOD* gene was performed with the cDNA library as the template and the following primer combinations: SOD3F and SOD4R, SOD5F and SOD6R (Table 2.2), and TRAP C1 control primers (EtV and EtZ, Spano, *et al,* 1998(b)). Following confirmation of the presence of the *SOD* gene within the library, PCR amplification was performed with M13 vector specific primers and *SOD* gene specific primers; SOD3F with M13rev and M13(-20), SOD4R with M13rev and M13(-20), SOD5F with M13rev and M13(-20), and SOD6R with M13rev and M13(-20).

### 2.2.7 Screening the cDNA λZAP library

#### 2.2.7.1 Measuring the Titre of the Phage Library

The titre of the phage library was determined by the infection of XL1-Blue MRF' strain of *E.coli* (Stratagene). The *E.coli* were grown in 10ml of LB broth supplemented with tetracycline to a final concentration of 10μg/ml. The resulting broth was streaked onto a LB tetracycline (10μg/ml) plate. A single colony was picked and LB broth, supplemented with magnesium chloride and sucrose to a final concentration of 10mM and 0.2%, respectively, was inoculated and incubated at 25°C overnight.

Serial dilutions of the phage were prepared in SM buffer (0.1M NaCl, 0.01M $MgSO_4 \cdot 7H_2O$, 0.05M Tris-HCl (pH 7.5), 5ml 2% (w/v) gelatin) from an estimated quantity of $1x10^5$ phage/μl to 1 phage/μl. XL1 cells, $OD_{600}$=0.5 in 600μl of 10mM $MgSO_4 \cdot 7H_2O$, were infected with a 10μl aliquot of the phage dilutions and incubated at 37°C for 15min. Large NZY agar plates (132mm diameter) were prepared for the titration. The infected *E.coli* cells were added to 7ml of NZY top agar, which had been melted and cooled to 55°C, and poured swiftly over the NZY plates. The number of phage plaques was counted from each plate, following an overnight incubation at 25°C, and the titre of the library was measured at $2x10^6$ phage/μl.

### 2.2.7.2 Colony Lift and Hybridisation of Phage Library: First Screen

The plates were prepared for colony lifts by placing at 4°C for 30min. A 132mm disc of Hybond-$N^+$ (Amersham) was placed on each plate and orientated by piercing with a pin. After 30min at RT, the membrane was removed and placed in denaturation solution (0.5N NaOH, 1.5M NaCl) for 15min. This was followed by a 15min incubation in neutralisation solution (0.5M Tris-HCl, 1.5 M NaCl). The membranes were then rinsed briefly in 2X SSC and allowed to dry before fixing the DNA by UV crosslinking. Hybridisation proceeded with a $^{32}P$-labelled probe, as described in section 2.2.4.

Following overnight exposure to photographic film, positive plaques were identified by aligning the developed film against the plates according to the orientation of the pin points.

The plaques were then cored from the plates and the phage were suspended in 3ml of phage buffer (0.01M Tris-HCl (pH 7.5), 0.01M $MgSO_4 \cdot 7H_2O$) overnight with agitation.

### 2.2.7.3 First Subscreen of Selected Plaques

A total of eight plaques were selected following the first screen of the library. Dilutions of the phage suspensions (Section 2.2.7.2) were prepared; 1:1, 1:10 and 1:100 dilutions in SM buffer. The stage of the screen employed smaller culture plates with 300µl of XL-1 cells infected and plated in 3ml of NZY top agar. The colony lift, hybridisation and plaque selection (Section 2.2.7.2) was completed on the 1:1 and 1:10 dilutions.

### 2.2.7.4 PCR Amplification of *SOD* from Selected Phage

Twelve plaques in total were selected from the first subscreen and eluted in 1.5ml of phage buffer, as described previously. DNA was extracted from the phage lysate by phenol/chloroform extraction, as indicated in section 2.2.6. PCR amplification with *SOD* specific primers confirmed the presence of *SOD*; SOD3F and SOD4R with phage lysate and extracted DNA as template, SOD7F and SOD8R and M13rev and M13(-20) with extracted DNA. A second amplification was performed with the SOD7F and SOD8R reaction, which involved using 1µl of PCR product from the initial amplification reaction as the template with all other conditions remaining unaltered. The PCR products were analysed further by Southern blot and hybridisation with a radiolabelled *SOD* specific probe, as described in section 2.2.4.

### 2.2.7.5 Excision of Selected pBluescript Phagemid

Four samples were selected for the excision of the pBluescript phagemid which required ExAssist® Interference-Resistant Helper Phage and SOLR™ Strain of *E.coli* (Stratagene). The SOLR™ cells were grown on LB agar supplemented with kanamycin to a final concentration of 50μg/ml at 30°C. XL1-Blue MRF' cells were prepared as described previously in section 2.2.7.1. The *E.coli* cells were resuspended in 10mM $MgSO_4$ at an $OD_{600}$ of 1.0. The XL1 suspension (200μl) and 250μl of the phage stock was incubated with 1μl of ExAssist® Helper Phage at 37°C for 15min. LB broth (3ml) was added to each infection and they were then incubated at 37°C with agitation for 3hr. The tubes were heated at 65°C for 20min and then centrifuged at 10000xg for 15min. The supernatant, containing the excised phagemid, was removed and this stock was stored at 4°C.

To obtain a single clone of the excised phagemid, two dilutions of the stock; 1:1 and 1:10, were used to infect 200μl of SOLR™ cells, which were then incubated at 37°C for 15min. The infected cells (200μl) were plated on LB-ampicillin agar plates (50μg/ml) and incubated at 37°C overnight. A single colony was selected from each 1:10 dilution plate and used to inoculate LB liquid medium supplemented with ampicillin (50μg/ml) and grown with agitation at 37°C overnight.

The pBluescript phagemid containing the clone insert was isolated by resuspending the bacterial pellet in 350μl of STAT buffer (0.1M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 5% Triton X-100) and 35μl of lysozyme. Cell lysis progressed for 60min

at 100°C and the proteins and cell debris were then removed by centrifugation at 20000xg for 10min. The DNA was precipitated by the addition of isopropanol (1:1, v/v) and 3M sodium acetate, pH5.2 (1:10, v/v) and pelleted by centrifugation at 20000xg, 4°C for 15min. The dry DNA pellet was resuspended in 100µl $H_2O$. The insert was mapped by exonuclease restriction enzyme digestion. A double digest with *Xho*I and *Not*I released the insert and revealed it's full length. *Eco*RI is known to cut within the determined *SOD* sequence and was used alone and also with *Xho*I to determine which inserts potentially contained the *SOD* gene. From this, four samples were selected for sequencing at Molecular Biology Support Unit (MBSU), University of Glasgow. The sequences were analysis as indicated in section 2.2.3.2.

### 2.2.7.6 Second Subscreen of Selected Plaques

A second subscreen proceeded on a total of 12 plaques selected from the initial screen. Colony lifts, hybridisation, plaque selection, phagemid excision and restriction digest analysis were completed as described previously. From this, a second batch of phagemid was selected for sequencing at MBSU, University of Glasgow. The sequences were analysed as described in section 2.2.3.2.

### 2.2.8.1 Isolation of RNA from Sporozoite Suspension

Total RNA was isolated from *C.parvum* oocysts. Sporozoites were isolated from approximately $8x10^6$ oocysts (approximately $3.2x10^7$ sporozoites). To release the sporozoites, the oocysts were pelleted by centrifugation at 3000xg and resuspended in

400µl of PBS. Four 100µl aliquots were prepared and the oocysts were incubated at 37°C for 2h with 10µl of 15% taurocholic acid. The sporozoites were recovered by centrifugation and resuspended in 3ml of TRI REAGENT™ (Sigma). Cellular proteins and high molecular weight DNA were removed by additional centrifugation at 12000xg for 10min at 4°C. The sample was allowed to stand a RT for 5min before the addition of 200µl of chloroform. The contents were mixed thoroughly by vigorous shaking and then allowed to settle at RT for 15min. Three phases containing RNA, DNA and protein were separated by centrifugation at 4°C for 15min. The aqueous phase containing the RNA was removed, isopropanol (1:10, v/v) was added and any remaining DNA was removed by centrifugation at 12000xg for 10min at 4°C. Isopropanol (500µl) was added and, following 10min at RT, the RNA was pelleted by centrifugation at 12000xg for 10min at 4°C. The RNA pellet was washed with 3ml 75% EtOH, vortexed and centrifuged at 12000xg for 5min at 4°C. The pellet was allowed to dry before being resuspended in 100µl of diethyl pyrocarbonate (DEPC)-treated $H_2O$. This RNase-free water was prepared by adding 10% (v/v) DEPC in ethanol to 990ml of $H_2O$ and incubating overnight.

The RNA was visualised on a formaldehyde gel. The preparation of solutions and methods for the electrophoresis of RNA through gels containing formaldehyde were followed as described by Sambrook *et al* (1989).

#### 2.2.8.2 5'RACE

The unknown sequence remaining at the 5'region of the *SOD* gene was investigated by 5'RACE. The 5'/3'RACE Kit (Roche UK Ltd) was used for amplification of cDNA from the internal known *SOD* sequence. One new primer, INV1, was required for this application and was used in conjunction with SOD4R and SOD6R. All primer positions are indicated in figure 2.1.

## 2.3 Results

### 2.3.1 Sequence Analysis of *SOD* PCR product from *C.parvum* Type II Genomic DNA

#### 2.3.1.1 Nucleotide sequence analysis

The initial approach of PCR amplification in cloning the *SOD* gene from Type II genomic DNA was optimised for the degenerate primers FCRP-SOD and RCRP-SOD. Controls included amplification of *T.gondii* DNA with the degenerate SOD primers, *Cryptosporidium parvum* DNA with COWP primers and *Toxoplasma gondii* DNA with actin primers. The optimal annealing temperature was established using the Robocycler (Stratagene), which allowed for simultaneous amplifications with a gradient of annealing temperatures. This indicated that the optimal annealing temperature for the degenerate SOD primers with *T.gondii* DNA was approximately 50°C. Following this, a range of *C.parvum* DNA samples were tested and this resulted in amplification of a product of 434bp

from *C.parvum* genomic DNA. The fragment was cloned for sequencing as described in section 2.2.3.1.

Sequence analysis of the PCR product confirmed that the *SOD* gene had been successfully amplified from *C.parvum* genomic DNA. The sequence obtained was entered into the ORF program (NCBI). The largest open reading frame identified was 381 base pairs in length. This sequence was then entered in the BLAST program to search for homologies within the nucleotide database at NCBI. The results obtained from this BLAST search showed that the query sequence displayed highly significant similarity with the *P.falciparum* FeSOD sequence (accession number Z49819), showing 52% identity with 69% positive matches. The *C.parvum* sequence showed highly significant similarity to other superoxide dismutase sequences including those from such diverse organisms as a number of *Plasmodium* species, *Leishmania* sp., *Legionella pneumophila, Helicobacter pylori, Photobacterium leiognathi* and *Arabidopsis thaliana* (Bécuwe, 1996)

The *C.parvum SOD* nucleotide sequence identified at this stage was incomplete, as the degenerate primers had been designed to amplify an internal region of the *SOD* gene. The alignment of the *C.parvum SOD* amino acid sequence with *SOD* gene sequences from other protozoa gave an indication of the length of the remaining sequence (Figure 2.3). Although the size of the *SOD* gene of other species ranges from ~600bp to ~1000bp, the size of the gene in the more closely related species of *P.falciparum* and *T.gondii* suggested that there were approximately 80bp at the 5' end and a further 115bp at the 3' end of the *C.parvum* sequence which remained to be characterised.

**Figure 2.3**

**Figure 2.3 Alignment of Fe-dependent Superoxide Amino Acid Sequences from Protozoa.** The alignment of Fe-dependent superoxide dismutase sequences of *C.parvum* with other protozoa highlighted the homology of the isolated sequence and also gave an indication of the portion of sequence remaining uncharacterised from the *C.parvum* gene. The other *FeSOD* sequences were obtained from the Genebank database accessible from NCBI. Accession numbers: *Toxoplasma gondii* acc.no. AF029915 and *Plasmodium falciparum* acc.no. Z49819.

### 2.3.1.2 Amino Acid sequence analysis

The amino acid sequence of the *SOD* gene was also considered and the 145 amino acid translation of the cloned fragment of the *C.parvum SOD* sequence was aligned with the amino acid sequence of the *P.falciparum FeSOD* gene (Figure 2.4). This alignment allowed for the identification of highly conserved regions involved in the metal co-factor binding and the regions that are characteristic of iron-dependent superoxide dismutase (Baert, *et al*, 1999(a)) and are highlighted in figure 2.4.

This sequence alignment suggests that the *C.parvum SOD* gene identified is most likely to code for an iron-dependent superoxide dismutase. All regions required for the distinction between the different types of SOD, as indicated by Baert *et al* (1999(a)), were present in the internal fragment of the gene. Two amino acids substitutions were present within the *C.parvum* sequence in these recognised conserved regions of the *SOD* gene. Examination of the primary structure at these positions revealed that the altered amino acid sequence was due to a single nucleotide change in both cases.

**Figure 2.4**

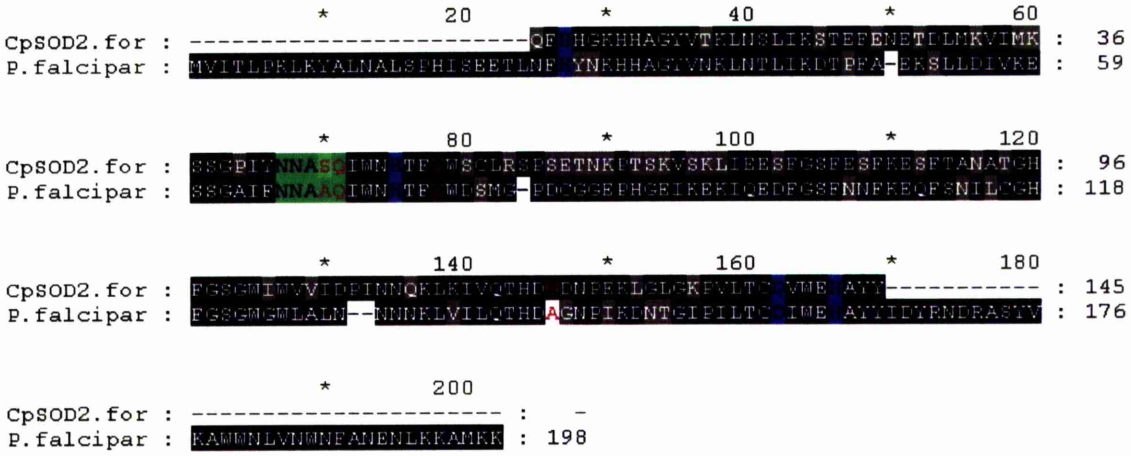


**Figure 2.4 Amino Acid Sequence Alignment of *C.parvum SOD* with *P.falciparum FeSOD*** Highly conserved regions highlighted in the *superoxide dismutase* gene sequence of *P.falciparum* (Baert, *et al*, 1999(a)) were employed to determine the type of *SOD* gene identified from the *C.parvum* genome. These criteria were as follows: four highly conserved metal binding ligands His-27, His-74, Asp-158 and His-162 (blue background), a distinctive region between positions 60-77 N-N-A-A-Q in FeSODs and F/N-N-G/A-G-G in MnSODs (green background), and four amino acids conserved in FeSODs Ala-69, Gln-70, Tyr-77 and Ala-142 (red text). Two of the conserved residues were modified in the *C.parvum* sequence: Ala-69 and Ala-142 to Ser and Gly, respectively, and were both due to a single nucleotide change at both positions.

### 2.3.2 Analysis of a PAC Genomic Library

Successful cloning of the central region of the *C.parvum SOD* gene provided a suitable template for the production of a highly specific probe for Southern hybridisation. This facilitated screening of the *C.parvum* genomic PAC library (HGMP). The initial hybridisation of the PAC library filter resulted in the identification of a single clone. As the clones were spotted onto the filter in duplicate, each positive signal highlighted two colonies and the duplicated colonies were arranged according to a specific grid pattern allowing for identification of each individual clone number. The pattern observed from two positive colonies, highlighted by the DIG-labelled probe, indicated that clone number 10-H11 contained the *SOD* gene. This result was reproduced with a second filter, thus confirming the identification of a single specific clone.

Clone 10-H11 was obtained from HGMP and prepared for DNA analysis (Section 2.2.5.2). Unfortunately, the preparation of the high molecular weight PAC DNA proved difficult. The recommended preparation protocol produced a very low yield of DNA, as did preparative commercial kits specifically designed for the isolation of high molecular weight DNA. As a result, further analysis of the insert was limited, although, confirmation of the presence of the *SOD* gene was obtained by PCR amplification with *SOD* gene specific primers (Section 2.2.5.4 and Figure 2.5).

Vector specific primers were designed to the sequence flanking the insert and PCR amplification from the 10-H11 clone was achieved with *SOD* specific primers and vector specific primers. Although a product was obtained from SOD5F and both vector specific

primers (Figure 2.6), Southern hybridization and sequence analysis confirmed that only one primer combination gave amplification of the *SOD* gene from within the insert to the arm of the vector. This product only provided additional 32 base pairs of 3' sequence of the *SOD* gene as the sequence of interest lay directly at the junction of the cloned insert and the vector (Figure 2.7).

Long template PCR was used as an additional method to amplify the large insert of the PAC clone. Following extensive troubleshooting, the amplification failed to yield a result.

In parallel, an approach to subclone the PAC insert proceeded. Fully digested PAC DNA was analysed by Southern hybridisation with a *SOD* specific probe (Section 2.2.5.3). Although numerous attempts were made, no hybridization signal was obtained. This prevented the subcloning of the insert DNA.

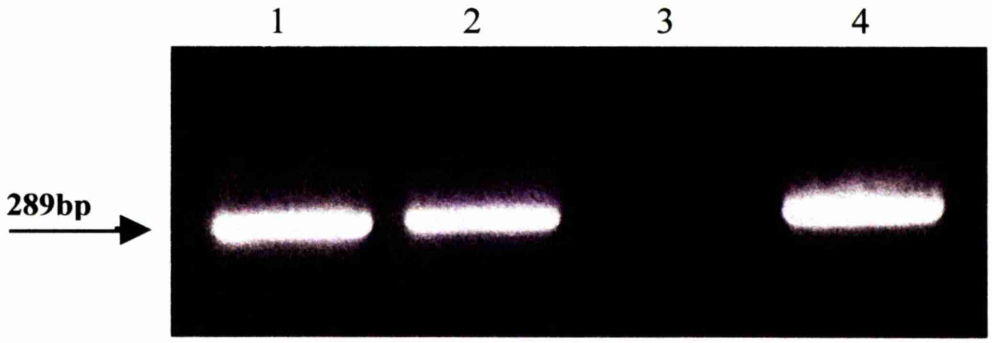



**Figure 2.5 PCR amplification of the *C.parvum SOD* gene from PAC clone 10-H11.** Lanes one and two show the 289bp PCR product amplified from the PAC clone with SOD3F and SOD4R gene specific primers (figure 2.1 and table 2.2). Lane three show the negative control with water substituting the template DNA and lane four shows the positive control with amplification from the SOD clone (section 2. ) and the SOD3F and SOD4R gene specific primers.

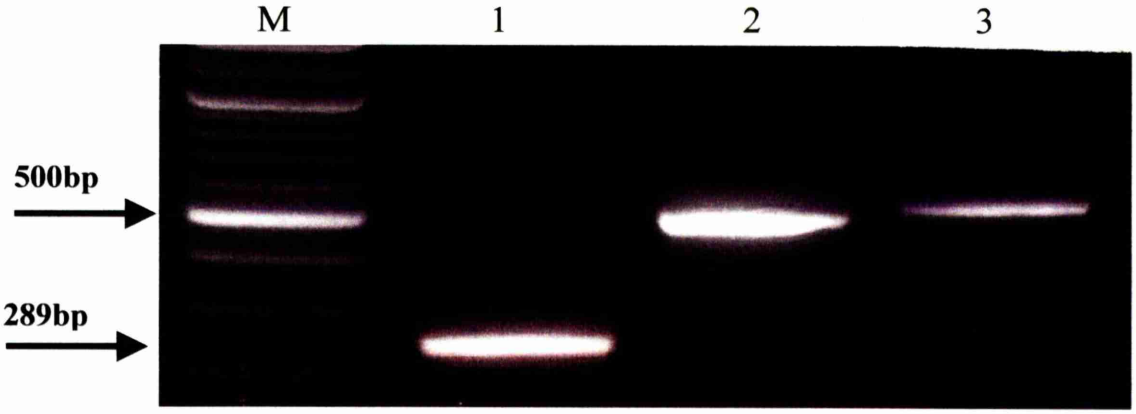



**Figure 2.6 Amplification of the *SOD* gene from the PAC clone with *SOD* specific and vector specific primers.** The *SOD* gene was amplified from the PAC clone the (1) SOD5F and SOD6R, (2) SOD5F and CYP1F and (3) SOD5F and CYP2R (Figure 2.1 and Table 2.2). The products obtained form reaction 2 and 3 were sequenced and this confirmed that only reaction 2, that is, amplification with the *SOD* specific primer and CYP1F vector specific primer, was specifically amplifying the *SOD* gene from the PAC clone.

**Figure 2.7**

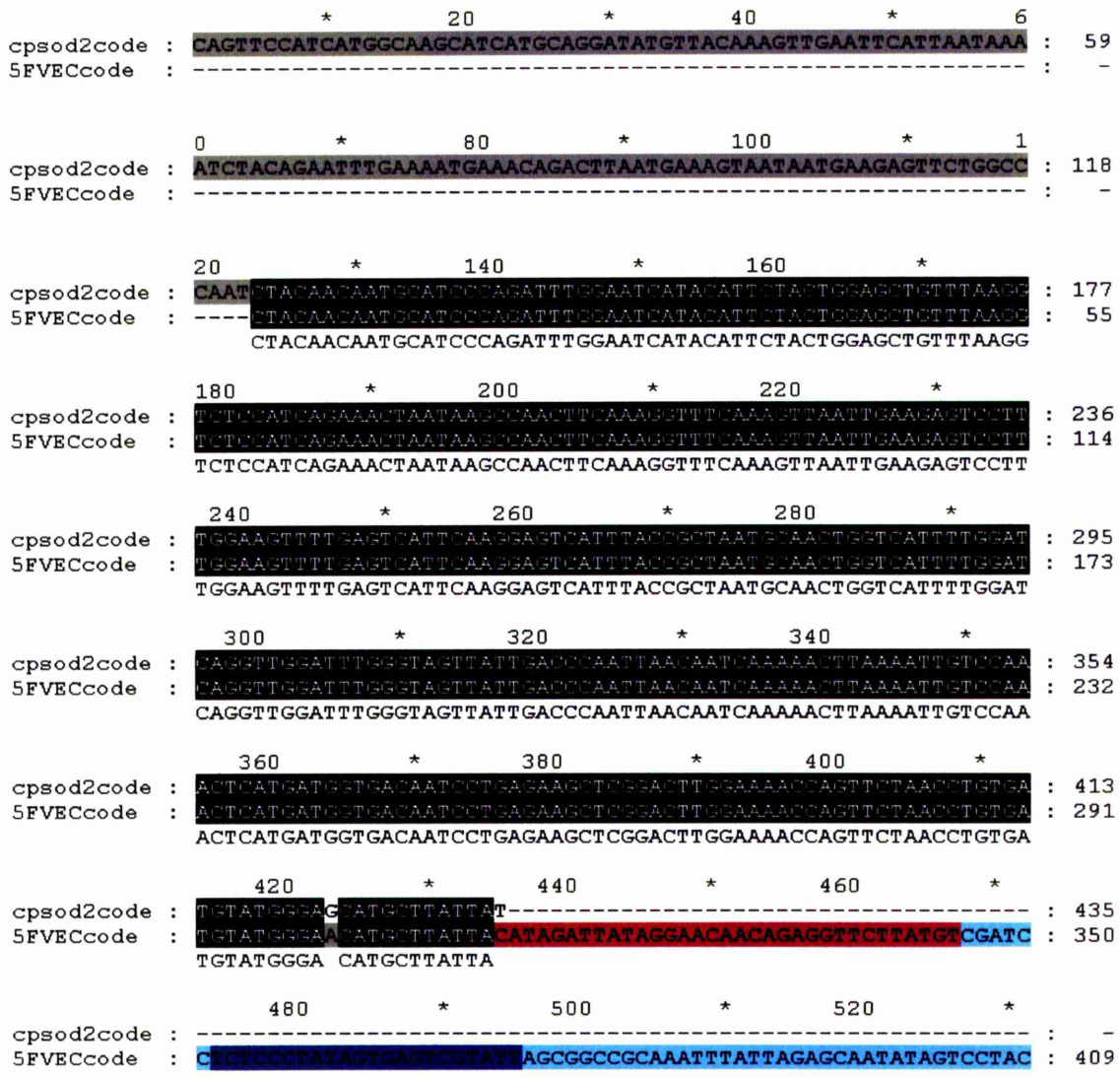



**Figure 2.7 The sequence of the internal fragment of the *SOD* gene isolated from *C.parvum*** The total sequence identified from the *SOD* gene of *C.parvum*. Sequence cpsod2code was identified by PCR amplification from *C.parvum* genomic DNA with degenerate primers. The 5FVEC sequence was obtained by amplification from the PAC clone with *SOD* specific and vector specific primers. The 5FVEC sequence shows 32bp of novel sequence (red) of the *SOD* gene and the T7 vector primer (dark blue) within the 10-H11 vector sequence (pale blue), displaying the position of the *SOD* gene in relation to the vector arm.

In addition, inverse PCR was employed to amplify the flanking regions of the known *SOD* sequence within the 10-H11 PAC clone. Unfortunately, this method was also unsuccessful in yielding a result.

### 2.3.3 The cDNA λZAP Library

An initial approach to amplify the *SOD* gene by PCR from a cDNA library confirmed the presence of the gene within the library and also provided evidence that the superoxide dismutase gene is expressed during the sporozoite developmental stage of *C.parvum*. It was also suggested at this stage, that the *SOD* gene may have been strongly represented within the library, as the comparative levels of PCR product between the *SOD* gene and the TRAP C1 control indicated. The TRAP C1 gene had been originally isolated from this cDNA library (Spano, *et al*, 1998(b)).

The presence of the *SOD* gene within the library was confirmed by amplification of an internal fragment of the gene with specific primers, thus, offering the library as a viable source for identification of the remaining *C.parvum SOD* sequence. Vector specific primers were employed together with *SOD* specific primers to amplify from within the known *SOD* gene sequence to the vector sequence adjacent to the insert. Extensive primer combinations were included to maximize the likelihood of successful amplification, despite the library construction employing directional cloning techniques (Spano, *et al*, 1998(b)). Sequence analysis of seven PCR products obtained by this method showed that the amplification was not specific and this PCR approach was discontinued.

It was then decided that a screen of the library would be necessary to proceed with the cloning of the full coding sequence of the *SOD* gene. Subsequent to the determination of the library titre, the first screen of the library was initiated (Section 2.2.7.2). A total of 8 plaques were isolated from the first screen and the phage were recovered for further selection. As the phage plaques were relatively dense, a subscreen was required to separate and isolate single positive phage plaques. Following this selection stage, 12 plaques were isolated and the phage were recovered.

As the number of potential positive phage was reasonably high, further selection was required before the extensive process of phagemid excision proceeded. DNA was extracted from the 12 phage lysate suspensions for PCR amplification with *SOD* specific primers: with SOD7F + SOD8R and SOD3F + SOD4R and also with M13 primers. The lysate was also used as template for PCR amplification without further isolation of the DNA. The products from these amplification reactions were analysed by Southern blot and hybridization with a $^{32}$P-labelled *SOD*-specific probe. From this analysis four samples were selected for excision of the phagemid (Section 2.2.7.5).

Following the excision reaction, five colonies were selected from each four samples and the pBluescript phagemid DNA was recovered and analysed by endonuclease restriction digestion. The presence of an insert was initially verified by digesting the DNA with *Xho*I and *Not*I. The restriction enzyme *Eco*RI is known to recognize at least one site within the *SOD* gene and was used to give an indication of the presence of the gene within each clone. As the full sequence of the insert DNA was unknown, the restriction patterns resulting from these digests were not conclusive, however, it was possible to suggest at this stage that at

least two of the clones may have contained the *SOD* gene. One clone from each sample was selected and both strands of the insert were sequenced using vector specific primers.

Following this extensive and thorough selection process, the sequence results revealed that none of the selected clones contained the *SOD* gene. These results, however, were not reliable as a high background signal prevented interpretation of the results from two samples and the third and fourth samples gave very different sequences from their complementary strands, suggesting contamination with foreign DNA. These clones had been transformed into an *E.coli* JM109 cell line for long-term storage of the clones. This allowed for the efficient isolation of clones for a further attempt at sequencing the insert. The results from this second round of sequencing confirmed that these clones did not contain the *SOD* gene. Sequence analysis revealed homology with ribosomal RNA (rRNA) sequences, which can result from contamination of the messenger RNA (mRNA) during construction of the library.

In parallel to this, a second subscreen proceeded with 11 phage plaques from the original subscreen. An identical screening process was employed as above, resulting in a further 7 samples being sequenced. The sequence analysis from these clones gave similar results as previously with homology to rRNA sequences and also with other *C.parvum* genes.

### 2.3.4 Southern Analysis

Southern analysis of the *C.parvum* genome was included to further characterise the *SOD* gene. Southern hybridisation involved digesting genomic DNA with four different restriction enzymes and fully separating the fragments on an agarose gel. The DNA was then transferred to a nitrocellulose membrane which was hybridised with a *SOD* specific probe. The results from this suggested that the superoxide dismutase gene is a single copy gene within the *C.parvum* genome (Figure 2.8).

### 2.3.5 5'RACE

The 5'RACE approach was employed to identify the remaining sequence to the 5' terminal of the gene. This method involves the production of cDNA by RT-PCR, attaching an anchor primer to the cDNA and then amplifying between this anchor primer and a gene specific primer. This protocol allows for stringent controls that can be included at each stage. Although several attempts were made, no result was obtained and, indeed, the products obtained from the controls did not correspond with the expected sizes. This method was not pursued further.

**Figure 2.8**

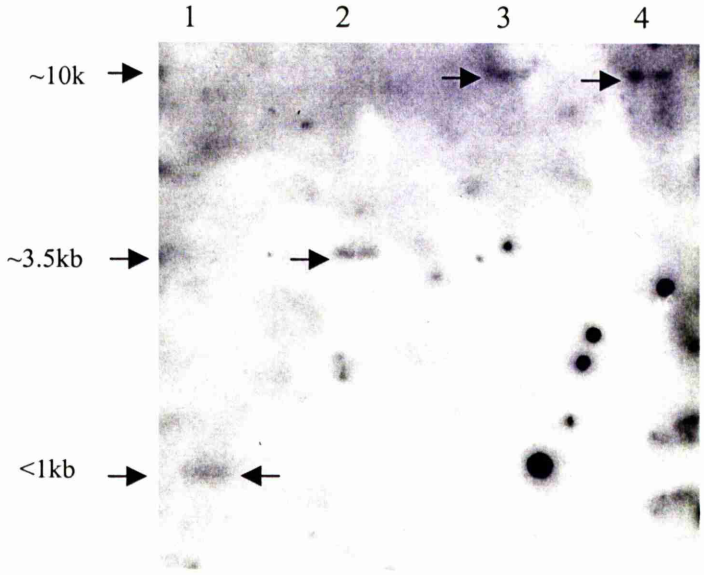



**Figure 2.8 Southern hybridisation analysis of the *C.parvum* genome.** *C.paruvm* genomic DNA was digested with four different restriction endonucleases: Lane 1 with *Eco*RI, Lane 2 with *Nsi*I, Lane 3 with *Sac*I and Lane 4 with *Xho*I. The enzymes used for samples one and two cut within the known SOD sequence and the enzymes used in samples three and four do not. The arrows indicate a single band from each sample following hybridisation with a *SOD* specific probe.

## 2.4 Discussion

The initial approach adopted for the identification of the *superoxide dismutase* gene was PCR amplification from genomic DNA with degenerate primers, designed according to the *SOD* sequence from *Plasmodium falciparum* and *Toxoplasma gondii* (Section 2.2.2 and Table 2.1). The primers were positioned in highly conserved regions within the *SOD* gene, as identified from an alignment from a range of species (Bécuwe, *et al*, 1996). This allowed for the amino acid sequence to be selected and the final nucleotide sequence was determined by considering specific features of the *C.parvum* genome, including codon usage, dinucleotide preference and trinucleotide repeats (Liu, *et al*, 1999). The resulting primer sequences were, therefore, optimal for targeting the *SOD* gene from *C.parvum* genomic DNA.

The PCR reaction was optimised for amplification with the degenerate primers by considering a range of annealing temperatures encompassing the calculated melting temperature of the primers. The inclusion of *Toxoplasma gondii* genomic DNA provided a useful control to indicate if the primers were able to produce a specific product during amplification. Having established the amplification parameters with *T.gondii* genomic DNA, a range of *C.parvum* DNA samples were tested with a series of dilutions in an attempt to obtain amplification of the *SOD* gene. A product of 434bp was obtained. This product was cloned, sequenced and identified as a gene fragment with high homology to *SOD* genes from other organisms including *P.falciparum* and *T.gondii* and, thus, likely to represent the homologous *superoxide dismutase* gene from *Cryptosporidium parvum*. This

central fragment of the *SOD* gene was to remain the majority of the sequence characterised during this project as only a further 32bp of the 3'terminal were obtained by PCR from the PAC genomic clone (Section 2.2.5.4). This sequence of 466bp was sufficient, however, to reveal important information about the gene.

There are three different forms of the *SOD* enzyme that are characterised by the metal co-factor present within the active site: MnSOD, Cu/ZnSOD and FeSOD. There are also highly conserved regions of the protein sequence which allow for distinction between the different types (Figure 2.4 and Baert, *et al*, 1999(a)). It was possible to infer from analysis of the *C.parvum* sequence that this was a Fe-dependent SOD.

There were, however, two conserved amino acids that were altered in the *C.parvum* sequence: Ala69 to Ser and Ala142 to Gly. At both positions, these altered amino acids were due to a single nucleotide change: G to T and C to G, respectively. These changes at the nucleotide level appear to represent accurately the *C.parvum* sequence as sequences obtained from a number of different *C.parvum* isolates, for the polymorphic analysis discussed in Chapter Three, show that these distinct nucleotides occur consistently in all isolates. Although these positions are known to be highly conserved between a variety of organisms, they are not present in the catalytic site of the enzyme or the residues involved in binding the metal cofactor. It is, therefore, possible that conservation of these amino acids does not extend to *C.parvum*. Further experiments on the protein activity would be necessary to confirm the type of *SOD* isolated by this approach. Differential sensitivity of the enzyme to certain compounds can be employed to investigate the type of SOD present in *C.parvum* (Section 1.3).

Following the identification of the partial fragment of the *SOD* gene from *C.parvum* genomic DNA, a suitable resource was required to identify and clone the remaining gene sequence. Initially, the ideal resource appeared to be a genomic PAC library which had been prepared at HGMP, University of Cambridge, for HAPPY mapping genome analysis (Piper, *et al*, 1998(a), Piper, *et al*, 1998(b), Ioannoun, *et al*, 1994) and has been made available for research purposes. This library provided a tool for simplified genome wide analysis. The primary steps of identifying a specific clone containing the *SOD* gene by hybridisation of the high density gridded filter of the library and confirmation of the presence of the gene within the clone by PCR, was relatively straightforward. However, further analysis of the high molecular weight PAC DNA, each clone with an estimated total size of approximately 56kb, proved to be more challenging.

Despite numerous attempts, it proved impossible to obtain sufficient DNA from the PAC clone, preventing further analysis of the insert with this approach. It is possible that the low yield obtained was due to instability of the PAC clone within the bacterial vector. It is also possible that the insert was not stable within the PAC vector and propagation of the bacteria for DNA isolation induced loss of the insert from the clone. Indeed, preparation of DNA from other clones in the library was possible using the same method, thus suggesting that this certain insert was particularly unstable. The combined size of the vector and insert was such that it is also possible the clone was precipitated and removed with the bacterial genomic DNA during sample preparation. Low DNA yield and loss of the insert could explain why the subcloning attempts where thwarted when the *SOD* gene was not identified from PAC clone digests by specific hybridisation (Section 2.2.5.3). Consequently, PCR was

the only method by which the *SOD* gene could be identified form the PAC clone and this method was used to proceed.

An attempt was made with the PAC clone to amplify by PCR from the known *SOD* sequence to the vector sequence, although it was not known in what portion, relative to the vector ends, the *SOD* gene fragment was located in the 38kb insert. The gene of interest was, however, in close enough proximity to the vector for successful PCR amplification. Although the complete gene sequence was not contained within this single clone, an additional 32bp of the 3' terminal of the gene were obtained by this approach.

In parallel, an attempt was made to amplify across the full length of the clone, and also from the known *SOD* sequence to the vector, employing the Expand™ Long Template PCR System (Roche) which was developed to amplify large fragments of DNA. This system required a stringent protocol and was accompanied by extensive guidelines and troubleshooting suggestions. However, this approach was unsuccessful, probably due to the location of the *SOD* sequence at the very end of the insert, so further amplification toward the 5'-end of the gene would require an extremely long template of up to 38kb.

The PAC clone was also used as the template for inverse PCR. This approach was hampered by the limited quantity of the PAC clone, as an initial amount of approximately 1μg of DNA was required. Although the DNA was quantified and diluted to optimise the ligation step, limited starting material and loss of DNA at previous stages prevented optimal ligation conditions and no product was obtained from the final PCR amplification. This

method was not pursued extensively, due to time constraints. In addition, the PAC clone may not have been ideal for this approach as the large size and possible instability of the clone may have hindered the numerous sensitive steps in the protocol.

The PAC library had initially appeared to be ideal for the above approaches as it allowed for simple selection of a single genomic clone containing the *SOD* gene. This reduced the size of the source DNA from 10Mb of the complete *C.parvum* genome to approximately 38kb contained within a single PAC clone. The relatively large size of the PAC clone, however, was in itself troublesome. With no progress being made on DNA preparation, the genomic PAC library was disregarded as a suitable resource for isolation of the *SOD* sequence.

The subsequent approach made use of a *C.parvum* cDNA library. Initially, PCR amplification was used to determine whether the *SOD* gene was represented within the library. A strong, reproducible signal was obtained from amplification with *SOD* specific primers and this lead to further attempt to amplify from the known *SOD* sequence to the vector, as was tried previously with the 10-H11 PAC clone. This amplification produced erratic results that were neither specific nor reproducible. This could be explained by the numerous clones present in the library that was used as the template for amplification. The vector specific primers would have been able to anneal to every clone and this may have resulted in simultaneous, random amplification. This prevented further use of PCR amplification from the cDNA library and it was, therefore, required to screen the library.

The cDNA library screen proved to be an extensive procedure for identification of a single clone containing the *SOD* gene. Following selection of phage plaques, additional steps of

PCR amplification, Southern analysis and restriction digest analyses of the DNA were included to select further for possible *SOD* gene containing clones from the library. The negative results from a total of eleven sequenced clones were unexpected as several selection stages had been included and each stage provided stringent selection parameters. The mRNA sequences retrieved from the cDNA clones could be explained by contamination during construction of the library. The positive results obtained throughout the selection process are harder to explain, however, as both PCR with *SOD* specific primers and hybridisation with a *SOD* specific probe indicated that these clones were strong candidates. Having reached the end with all the selected clones, continuation of the library screen would entail returning to the full library and begin the selection process once again.

In conclusion, it can be suggested that from the results presented above that the *superoxide dismutase* gene fragment isolated from *Cryptosporidium parvum* genomic DNA is likely to encode a Fe-dependant SOD. This would support the theory that *C.parvum* does indeed contain mitochondria, a debate that has arisen from the apparent lack of mitochondria from microscopy studies (Tetley, *et al*, 1998). As Fe-dependent SODs are associated with mitochondria, the presence of a Fe-SOD and of other genes encoding typical mitochondrial proteins (Riordan, *et al*, 1999) would contradict such conclusions.

The *SOD* gene appears to be a single copy gene, although further confirmation of this is required. The *SOD* sequence shows a high degree of conservation with *SOD* genes isolated from a range of different organisms although the region displays a degree of polymophism, which may disassociate this gene with other *SOD*s. The gene sequence also shows a high AT content of approximately 65%, which is typical of the *C.parvum* genome. It is also

conclusive that the *SOD* gene is expressed during the sporozoite developmental stage of the parasite as the gene can be amplified from the cDNA library, which was constructed from RNA isolated from *C.parvum* sporozoites.

# Chapter Three: Polymorphism analysis of *SOD*

## 3.1 Introduction

The number of studies investigating the *Cryptosporidium parvum* genome has grown rapidly in recent years. A greater understanding of the genome would contribute to a variety of aspects in parasite and, subsequently, disease detection and control. Early molecular studies confirmed two genotypes of *C.parvum*: Type I, which is infectious to humans only and Type II which is infectious to both humans and animals (Peng, *et al,* 1997, Spano, *et al,* 1997). As *C.parvum* oocysts are neither phenotypically nor morphologically distinct it is desirable to employ a molecular typing method that can distinguish between the individual *C.parvum* genotypes. This molecular approach is especially desirable in the case of *C.parvum* as no efficient *in vitro* cultivation method is available.

Molecular typing systems were first investigated using methods including random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and direct sequencing approaches (Caccio, *et al*, 1999, Spano, *et al*, 1997, Spano, *et al*, 1998(a), Spano, *et al* 1998(b), Widmer, 1998, Morgan and Thompson, 1998, Sulaiman, *et al,* 1998). These methods have been criticised for their limitations, such as inaccurately representing polymorphic regions as they rely on size separation by gel electrophoresis and for being labourious. More recent approaches include the study of microsatellite sequence analysis and single-strand conformation polymorphism (SSCP) analysis for the identification of marker sequences containing genotypically distinctive polymorphisms

(Aiello, *et al,* 1999, Feng, *et al,* 2000, Gasser, *et al,* 2001). These methods provide simultaneous multiloci analysis and high sensitivity, allowing for rapid and accurate analysis of numerous isolates.

These studies have unveiled the highly polymorphic nature of the *Cryptosporidium parvum* genome and have presented a number of candidate genes for genotypic differentiation (Rochelle, *et al,* 1999, Spano, *et al,* 1998, Caccio, *et al,* 1999). Sequence analysis of eleven human isolates, five bovine isolates and one cervine/ovine isolate suggests that the Fe-SOD gene identified from the *C.parvum* genome may also be a suitable candidate for polymorphic differentiation between genotypes.

The initial aim of identifying polymorphism in the *SOD* gene between genotypes of *C.parvum* involved isolating the gene from a range of isolates. The selected isolates provided a range of Type I and Type II genotypes, obtained from a variety of hosts. Following nucleotide sequence analysis to identify polymorphic position within the gene, the amino acid sequences from each isolate were analysed to identify any possible alteration of the secondary structure that may lead to functional differences between the SOD proteins of different *C.parvum* genotypes.

## 3.2 Materials and Methods

### 3.2.1 Preparation of *C.parvum* Cell Lysate

The preparation of *C.parvum* cell lysates was achieved as described in section 2.2.1 with the exception of the final DNA purification step by phenol/chloroform extraction. Following centrifugation to pellet the cell debris, the supernatant was removed and stored at -20°C. Cell lysates were prepared from a range of *C.parvum* isolates, including those selected for the ozonation treatment describe in Chapter 4 of this thesis (Table 3.1). The isolates were chosen to provide a broad range of both Type I and Type II genotypes from human and animal origin. Type I isolates were from human clinical cases of cryptosporidiosis sent to the Scottish Parasite Diagnostic Laboratory (SPDL). Type II isolates were from both human clinical infections and from clinical bovine infections collected from Scottish farms by the Scottish Agricultural Colleges Veterinary Service. The cervine/ovine isolate was commercially available from the Moredun Research Institute, Edinburgh.

**Table 3.1**

| *C.parvum* REFERENCE NO. | ORIGIN | GENOTYPE |
|---|---|---|
| 25 | human | I |
| 120 | human | I |
| 121 | human | I |
| 126 | human | I |
| 127 | human | I |
| 317 | human | I |
| 378 | human | I |
| 116 | human | II |
| 117 | human | II |
| 633 | human | II |
| 642 | human | II |
| 181 | bovine | II |
| 182 | bovine | II |
| 598 | bovine | II |
| 652 | bovine | II |
| 91019 | bovine | II |
| C3 | cervine/ovine | II |

**Table 3.1 *C.parvum* isolates included in the polymorphism study.** The *C.parvum* isolates were selected to give a range of both Type I and Type II genotypes. All oocysts were purified and the genotype was determined as described in sections 4.2.1 and 4.2.2. Cell lyaste was prepared from nine of the isolates by Janice Brock, Division of Infection and Immunity, University of Glasgow, UK.

### 3.2.2.1 PCR Amplification

PCR amplification was performed with oocyst lysate preparation as the template and SOD3F and SOD4R or SOD7F and SOD8R gene specific primers (Table 2.1 and Figure

2.2). Initially, a range of volumes of lysate was tested to identify the optimal amplification conditions. An initial denaturation at 94 °C for 5min was followed by 30 cycles of 50 sec denaturation at 94 °C, 50 sec annealing at 50 °C and 1min elongation 72 °C, followed by a final elongation period of 5 minutes. The PCR products were analysed by agarose gel electrophoresis. Very low yields of product were obtained from this reaction, therefore a second round of amplification was required. This was accomplished by using 1µl of the first round PCR product as template for the second reaction. All other parameters of the reaction remained unchanged from the first round amplification.

#### 3.2.2.2 PCR Product Preparation

The PCR products from the second amplification were purified for sequencing with the QIAquick PCR Purification Kit (Qiagen). In the event of low product yield, the fragments were cloned as described in section 2.2.3.1 for sequencing at MBSU, University of Glasgow. All other fragments were sequenced directly. Each product was sequenced in both sense and antisense directions.

### 3.2.3 Sequence Analysis

Sequence analysis employed BLAST at NCBI, Genedoc alignment program.

## 3.3 Results

### 3.3.1 Identification of Single Nucleotide Polymorphisms

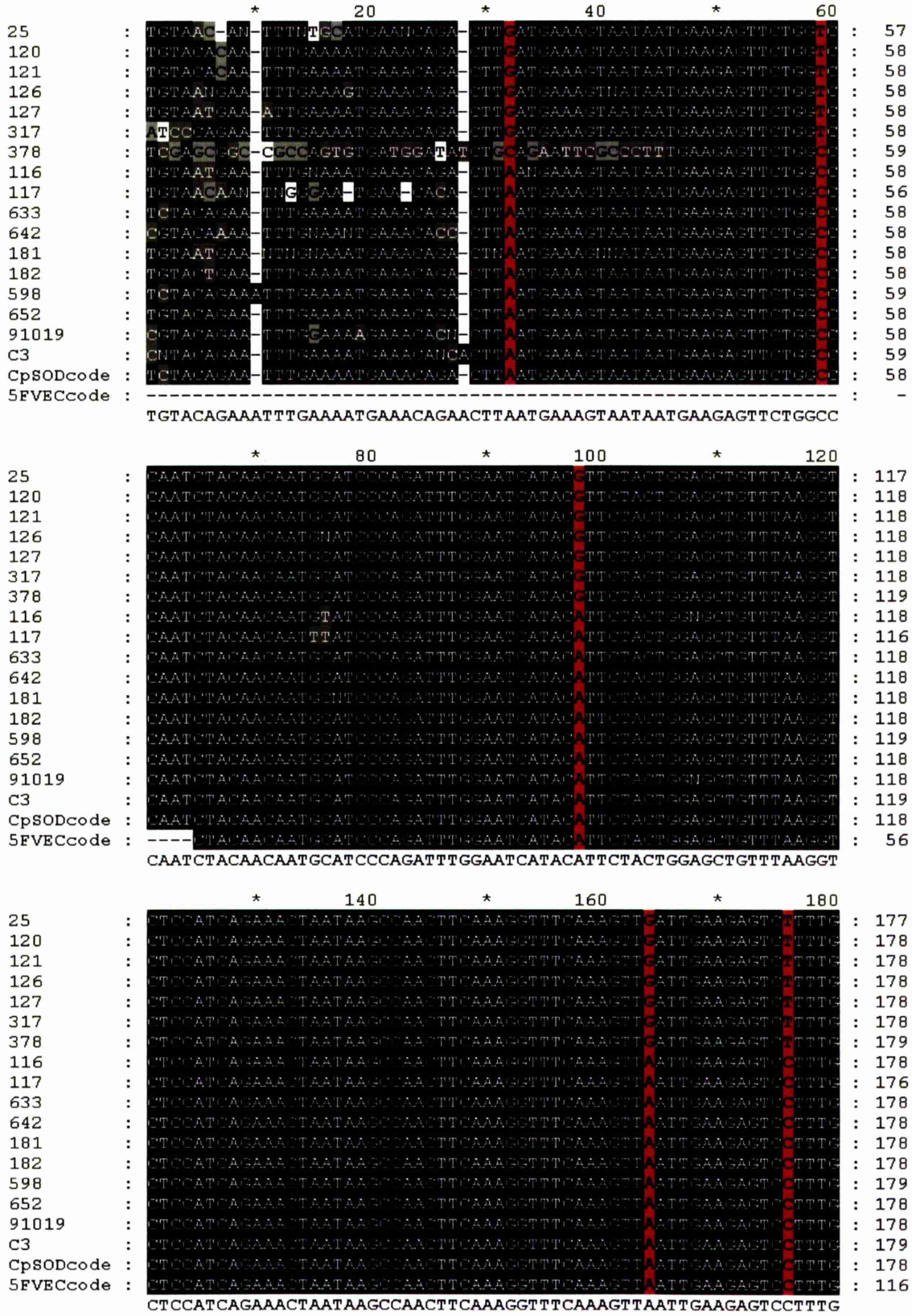
Analysis of the sequences obtained from the amplification of the *C.parvum* cell lysates indicated that there were a number of sites within the superoxide dismutase gene at which there was sequence variation of a single nucleotide between Type I and Type II isolates (Figure 3.1). There were six such positions within the 466bp fragment of the *C.parvum SOD* sequence. The alignment in figure 3.1 indicates a distinct clustering within each genotype at these polymorphic positions. Table 3.2 summarises these polymorphisms.

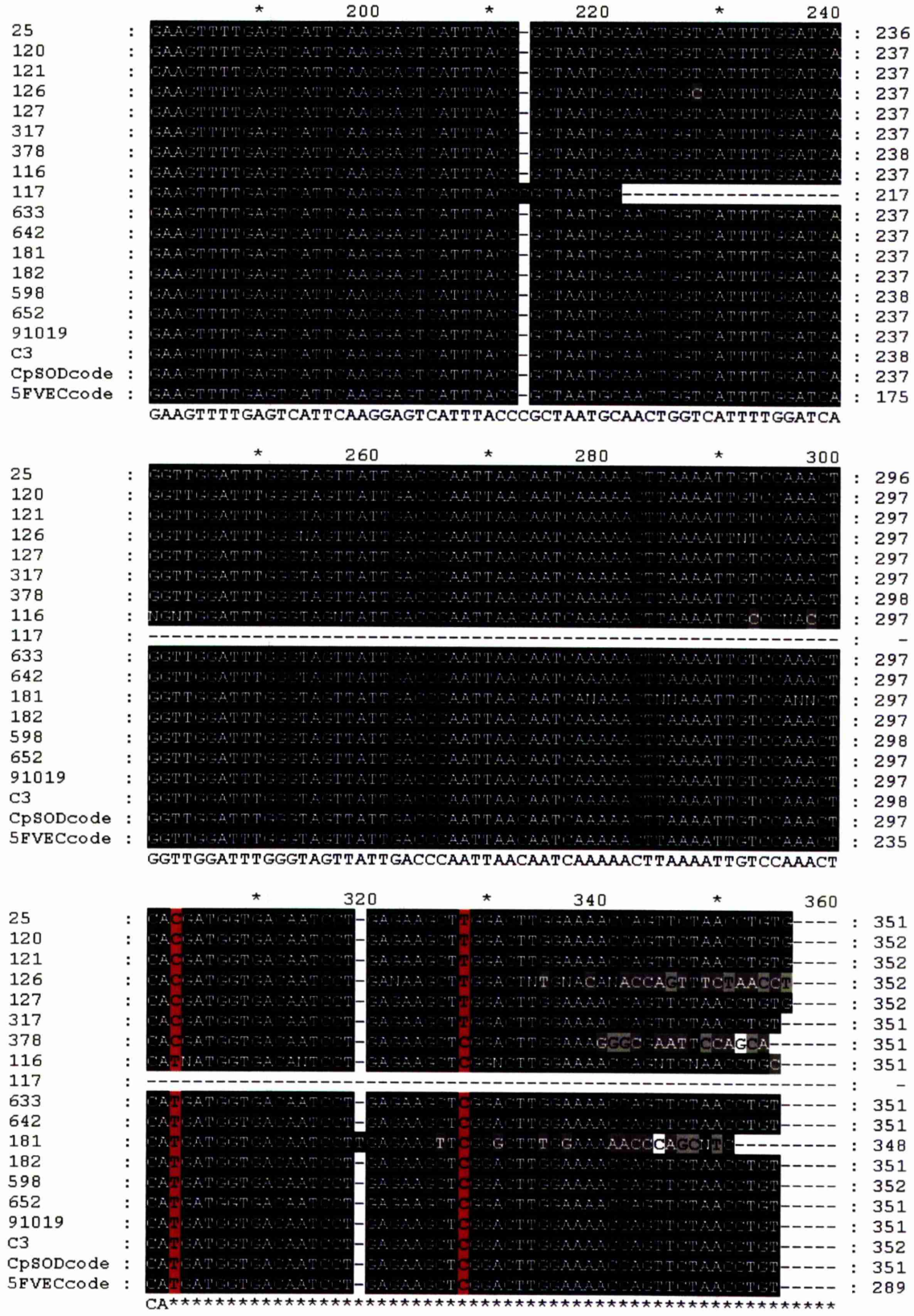
**Table 3.2**

| Position | Type I | Type II |
|---|---|---|
| 90 | G | A |
| 117 | T | C |
| 156 | G | A |
| 222 | G | A |
| 234 | T | C |
| 361 | C | T |

**Table 3.2 Properties of *C.parvum* SOD polymorphic nucleotides.** The positions of the polymorphic nucleotides are allocated according to the CpSODcode sequence shown in Figure 3.1.

**Figure 3.1**


25
120
121
126
127
317
378
116
117
633
642
181
182
598
652
91019
C3
CpSODcode
5FVECcode
TGTACAGAAATTTGAAAATGAAACAGAACTTAATGAAAGTAATAATGAAGAGTTCTGGCC
CAATCTACAACAATGCATCCCAGATTTGGAATCATACATTCTACTGGAGCTGTTTAAGGT
CTCCATCAGAAACTAATAAGCCAACTTCAAAGGTTTCAAAGTTAATTGAAGAGTCCTTTG


* 200 * 220 * 240
25 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 236
120 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
121 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
126 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGCCATTTTGGATCA : 237
127 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
317 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
378 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 238
116 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
117 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCGGGCTAATGC---------- : 217
633 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
642 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
181 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
182 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
598 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 238
652 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
91019 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
C3 : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 238
CpSODcode : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 237
5FVECcode : GAAGTTTTGAGTCATTCAAGGAGTCATTTACCG-GCTAATGCAACTGGTCATTTTGGATCA : 175
GAAGTTTTGAGTCATTCAAGGAGTCATTTACCCGCTAATGCAACTGGTCATTTTGGATCA
* 260 * 280 * 300
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120 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
121 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
126 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTNTCCAAACT : 297
127 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
317 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
378 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 298
116 : NGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGCCCAACT : 297
117 : ---------- : -
633 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
642 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
181 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCANAAACTTNAAATTGTCCANNCT : 297
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598 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 298
652 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
91019 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
C3 : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 298
CpSODcode : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 297
5FVECcode : GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT : 235
GGTTGGATTTGGGTAGTTATTGACCCAATTAACAATCAAAAACTTAAAATTGTCCAAACT
* 320 * 340 * 360
25 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGTG---- : 351
120 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGTG---- : 352
121 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGTG---- : 352
126 : CAGGATGGTGACAATCCT-GANAAGCTTGGACTTNTGNACANACCAGTTTCTAACCT---- : 352
127 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGTG---- : 352
317 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
378 : CAGGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAGGCCAATTGCAGCA------ : 351
116 : CANNATGGTGACAATCCT-GAGAAGCTTGGNCTTGGAAAACTAGTTCTAACCTGC----- : 351
117 : ---------- : -
633 : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
642 : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
181 : CATGATGGTGACAATCCTTGAGAATTCTGGTTTTGAAAACCCAGCTTG---------- : 348
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652 : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
91019 : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
C3 : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 352
CpSODcode : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 351
5FVECcode : CATGATGGTGACAATCCT-GAGAAGCTTGGACTTGGAAAACTAGTTCTAACCTGT----- : 289
CA**********

**Figure 3.1 Alignment of *C.parvum SOD* gene nucleotide sequences from Type I and Type II isolates.** The alignment of the *superoxide dismutase* gene sequence from a range of Type I and Type II isolates indicated that there are a number of polymorphic nucleotides between *C.parvum* genotypes

At each polymorphic position, the structure of the nitrogenous base remains unaltered. The pyrimidines, cytosine and thymine, alternate exclusively with each other, while adenine and guanine, the purines, are also exclusively exchanged between genotypes. It is also evident that every polymorphic nucleotide lies at the third position of the respective codon. This allows for the secondary structure of the protein to remain unchanged between genotypes (Figure 3.2) as the specificity of the codon for recognition of the complementary anticodon is maintained, even with a degenerate third base. One other important feature of the polymorphic nucleotides is that they are not present within the highly conserved regions of the superoxide dismutase gene. The amino acids required for metal cofactor interactions and those within the catalytic domains of the protein, as indicated in figure 2.4, remain conserved between genotypes.

**Figure 3.2**

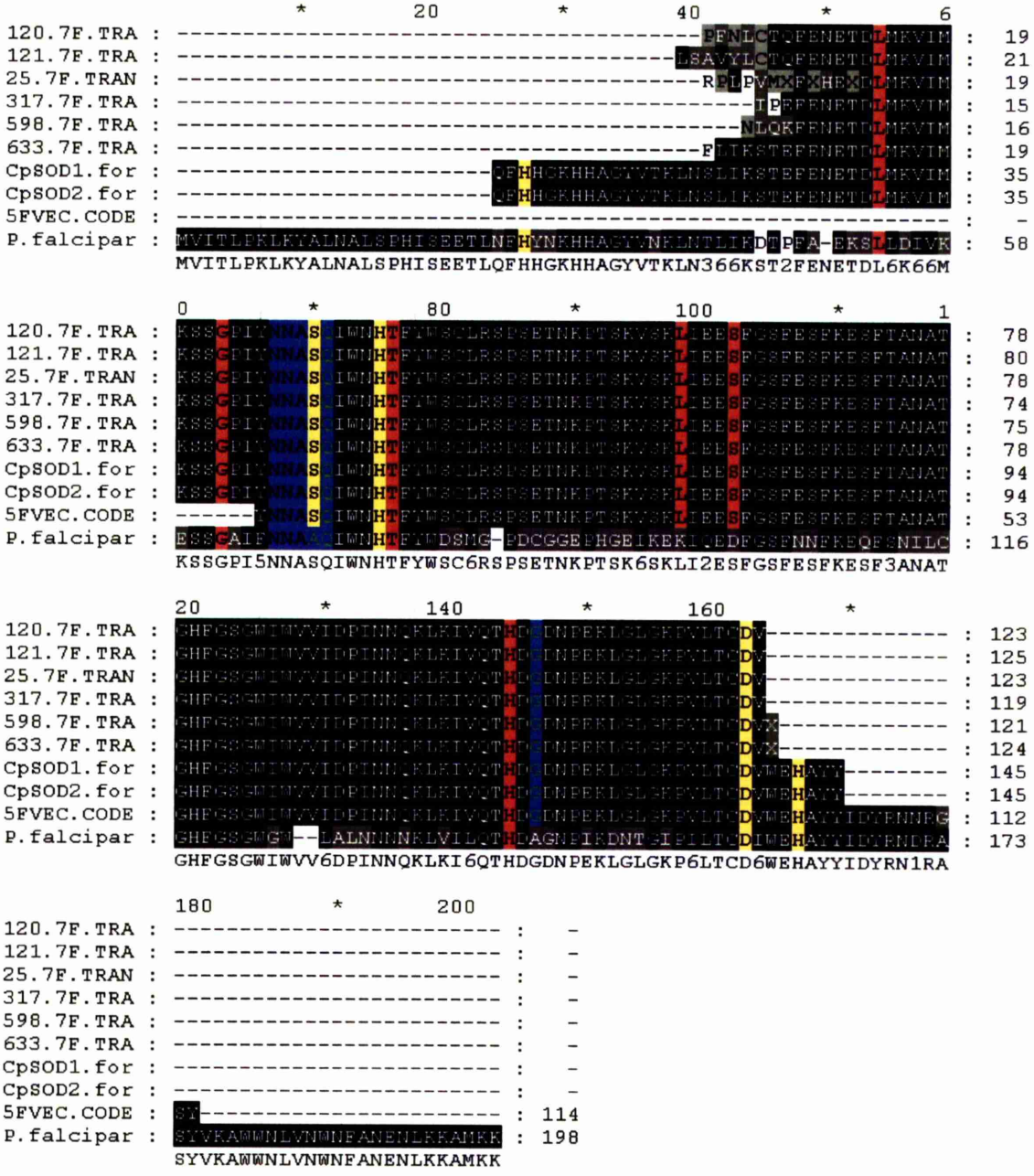

**Figure 3.2 Alignment of *C.parvum* SOD gene amino acid sequences from Type I and Type II isolates.** The amino acid alignment of the SOD gene from a range of Type I and Type II isolates shows that the polymorphic nucletides do not alter the secondary structure of the gene. The polymorphic positions are highlighted in red. It is also clear that the polymorphic nucleotides do not lie within the highly conserved region of the SOD gene.

## 3.4 Discussion

The polymorphic nature of the *C.parvum* genome has been documented previously (Peng, *et al*, 1997, Widmer, 1998, Aiello, *et al*, 1999, Feng, *et al*, 2000, Caccio, *et al*, 2000) and a number of candidate genes have been suggested for the differentiating between genotypes. Genotyping of *C.parvum* isolates is of utmost importance for the control of the pathogen as it would assist in understanding the genetic structure of polulations and provide a tool for tracing the routes of infection during cryptosporidiosis outbreaks. The significance of this is emphasized by the lack of a suitable preventative or therapeutic treatment for the infection.

The *superoxide dismutase* gene isolated from the *C.parvum* genome has revealed a number of polymorphic positions between Type I and Type II isolates. Within the partial fragment of the gene identified to date, there are seven positions that show distinct polymorphism between *C.parvum* genotypes (Figure 3.1). These polymorphic positions do not occur in the highly conserved regions of the gene and, as each polymorphism lies at the third wobble position of the codon, the amino acid sequence also remains unchanged (Figure 3.2). There are possibly more polymorphic sites within the *C.parvum SOD* gene that are located in the unidentified end fragments of the gene sequence.

There is potential for using this *SOD* gene for future genotyping analysis of *C.parvum* with particular significance for analysis of isolates from clinical cases of cryptosporidiosis. This loci, together with other identified genes showing polymorphism between genotypes, could

provide a suitable marker for rapid and accurate distinction between Type I and Type II *C.parvum* oocysts. The concept of developing a multiloci analysis tool for genotyping purposes will be strengthened by the identification of additional polymorphic markers, of which the *superoxide dismutase* gene is a strong candidate. However, the *SOD* gene fragment does not appear to be a suitable candidate for differentiating within Type I or within Type II isolates. For this, more discriminating markers are required, such as these offered by microsatellites and minisatellites.

# Chapter Four: Ozonation of *Cryptosporidium parvum* oocysts

## 4.1 Introduction

*Cryptosporidium parvum* oocysts are highly resistant to common disinfectants such as ammonia and bleach (Korich, *et al.*, 1990). Extremely high levels of these disinfectants are required to inactivate oocysts; these treatments are inappropriate and impractical for the large-scale disinfection of recreational and drinking water supplies. Numerous studies have considered the effectiveness of different disinfectants on the viability of *C.parvum* oocysts (Campbell, *et al,* 1982, Korich, *et al.,* 1990; Rennecker, *et al.*, 1999) and, as a result, ozone was considered the most effective, single disinfectant for *C.parvum* inactivation.

The present methods for water purification include coagulation, sedimentation and filtration to remove unwanted particles. Although this method is highly efficient in removing the majority if *C.parvum*, viable oocysts can survive this process and remain in the water. It is, therefore, necessary to identify an appropriate detergent that can be applied safely and economically to ensure inactivation of contaminating oocysts. It is possible that in swimming pools and bottled water production plants, where water volumes are relatively small and manageable, ozone disinfection of water could be employed practically and efficiently as an alternative to chlorination. Many European countries, including France, Germany and The Netherlands, currently use ozone for this purpose.

Ozone is produced as a natural consequence of metabolism in living organisms. High levels of free radicals, such as ozone, are damaging to the structure and content of cells and, as a result, defence against these reactive oxygen species (ROS) has evolved by means of antioxidant enzymes.

There have been a number of previous studies examining the effect of ozonation on the viability of *C.parvum* oocysts (Peeters, *et al*, 1989; Korich, *et al*, 1990; Finch, *et al*, 1993; Parker, *et al*, 1993, Rennecker, *et al*, 1999). These studies have included determining the optimal temperature, pH, contact time and concentration of ozone required for complete oocyst inactivation. However, the results of these studies have been inconsistent due to a number of factors including the use of different *C.parvum* isolates and the methods used for viability assessment. It has been suggested, however, that ozone has a diverse effect on oocyst viability with some isolates of *C.parvum* requiring greater levels of ozone for complete inactivation (Parker, 1993; Rennecker, *et al.*, 1999). The results of a previous study of the ozonation of *C.parvum* oocysts by J.Parker at the SPDL, Stobhill Hospital is shown in table 4.1. These results demonstrate a distinct variation in percentage viability following ozonation in a range of isolates from different hosts. No molecular typing system was available at the time of this study; therefore, the genotype of the oocysts was unknown.

**Table 4.1**

| Isolate | % Viability |
|---|---|
| cervine/ovine | 65 |
| human A | 52 |
| human B | 12 |
| bovine | 7 |

**Table 4.1 Ozonation of *C.parvum* oocysts** (Parker, 1993). The isolates included in this study display a clear variation in % viability following ozonation.

These observations were reflected by the work of Rennecker *et al,* (1999), who also reported different levels of resistance to ozone among different preparations of *C.parvum* oocysts. However, this study was limited to genotype II isolates from only two different sources and their diverse purification and storage conditions may have contributed to this variation. Differences in susceptibility of oocysts to ozone could present a real problem, where water purification depends on ozonation.

There was no effective molecular typing system for *Cryptosporidium parvum* at the time of the previously mentioned experiments, which would have allowed for the distinction between the Type I and Type II isolates. It is now possible to distinguish between isolates at the molecular level, allowing for the investigation of the possible correlation between the parasite genotype and susceptibility to ozone.

The aim of this study was to examine the effect of exposure to ozone on *C.parvum* isolates of known genotype under controlled and reproducible conditions and to determine whether differential susceptibility to ozone treatment exists between isolates of different genotype.

## 4.2 Materials and Methods

### 4.2.1 Oocyst purification

The oocysts were purified from fecal samples by employees at SPDL, Stobhill Hospital, Glasgow, UK.

### 4.2.2 Oocyst genotype determination

The genotyping of the isolates was determined by Miss Alison Shott, SPDL, Stobhill Hospital, Glasgow, UK, using the COWP and TRAP gene loci (Spano, *et al*, 1997, Spano, *et al*, 1998 (b))

### 4.2.3 Oocyst viability

#### 4.2.3.1 Microscopy

The initial viability of each isolate was assessed prior to ozonation. Oocysts were observed using an Olympus BH 2 microscope equipped with Nomarski differential interference contrast (DIC) optics to differentiate between intact oocysts containing sporozoites from

'ghost' shells which have lost their contents. This was complemented by epifluorescence microscopy to visualise specifically stained oocysts, prepared by the following method, which allowed for the distinction between viable oocysts, potentially viable oocysts and those that are non-viable.

#### 4.2.3.2 Oocyst labelling and viability determination

The inclusion or exclusion of two fluorogenic vital dyes was adopted as the method for determining viability of the oocysts. Each 100µl sample of approximately $1x10^5$ oocysts was prepared for labelling by incubating at 37°C in 1ml of acidified Hanks Balanced Salt Solution (HBSS) pH 2.75 for 1h. The oocysts were then washed three times with non-acidified HBSS (pH 7.2) by pelleting the oocysts, aspirating the supernatant to 20µl, and resuspending the oocysts in the remaining small volume. The Eppendorf was then filled with HBSS (pH 7.2) and the procedure repeated. After three washes with HBSS (pH 7.2) the oocysts were resuspended and the volume was adjusted to 100µl.

Oocysts were then incubated simultaneously with 10µl of the fluorogenic vital dyes 4',6-diamidino-2-phenylindole (DAPI), (working solution 2mg/ml in absolute methanol) and propidium iodide (PI), (working solution 1mg/ml in 0.1M PBS, pH 7.2) at 37°C for 1h 30min. Following incubation, 6µl of fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* monoclonal antibody (6x stock solution) was added and incubation at 37°C continued for a further 30min.

The stained oocysts were washed twice with HBSS as before and viewed by epifluorescence microscopy. Microscope slides were prepared by removing a 10μl aliquot of the oocyst suspension. The epifluorescence optics were used for the initial identification of the oocysts. This revealed the FITC antibody, which bound to the oocyst surface and appeared under fluorescence as a distinctive green halo of 4-6μm in diameter (Figure 4.1a); the expected size of a *C.parvum* oocyst. The DAPI stain was viewed by switching to the UV filter block (350-nm excitation, >450-nm emission) to reveal the nuclei, which appeared as a sky blue colour (Figure 4.1b). Switching to the green filter block (535-nm excitation, >590-nm emission) allowed visualisation of the PI stain, which appeared bright red (Figure 4.1c). Both the DAPI and the PI stain varied from distinct spots, which

**Figure 4.1**

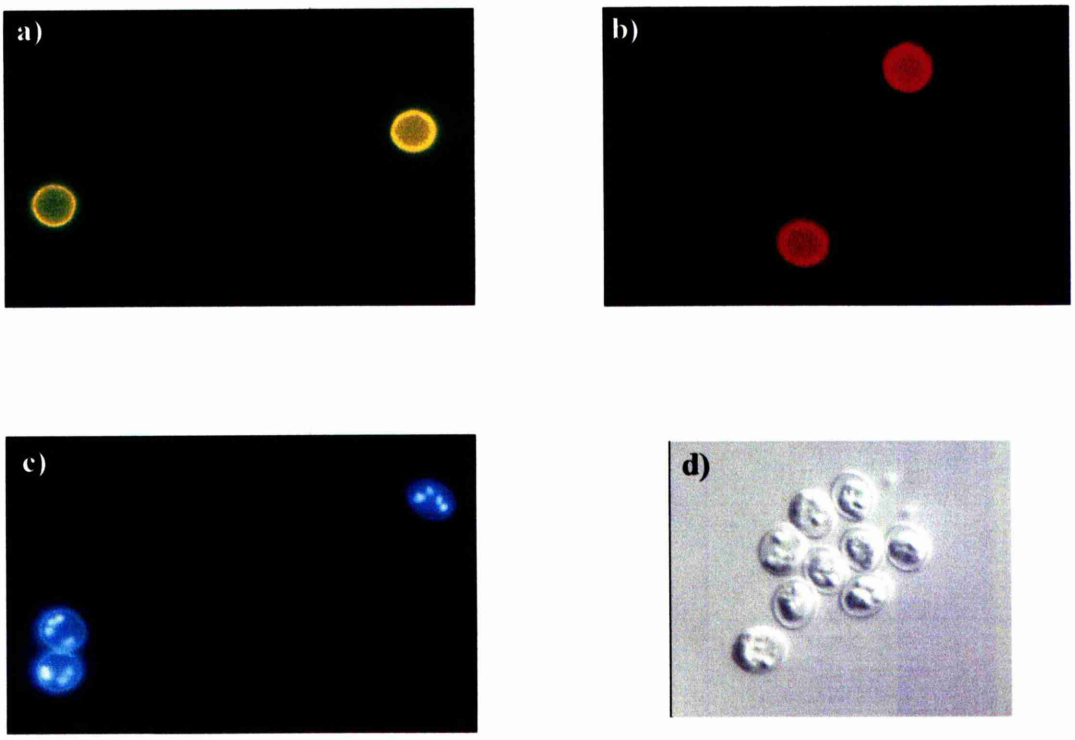


**Figure 4.1 *Cryptosporidium parvum* oocysts labelled with FITC antibody and stained with DAPI and PI.** (a) The FITC antibody appeared under fluorescence as a distinctive green halo and was employed to identify *C.parvum* oocysts. (b) The nuclei of sporozoites retained in viable and potentially viable oocysts were stained with DAPI. This fluorogenic vital dye was viewed under a UV filter block (350-nm excitation, >450-nm emission) and appeared as appeared as a sky blue colour. (c) The nuclei of non-viable sporozoites were also stained with PI. This fluorogenic vital dye was visualised under a green filter block (535-nm excitation, >590-nm emission) and appeared bright red. (d) *C.paruvm* oocysts viewed by Nomarski optics (figure 'd' only from http://www.biosci.ohio-state.edu/~parasite/home.html)

correlated with the position of the sporozoite nuclei, to a general fluorescence indicating more diffuse nuclear contents, suggesting a ruptured nuclear membrane. DIC optics were used to determine which DAPI$^-$ oocysts retained their contents without being successfully stained with DAPI. When this occurs, it suggests that these oocysts are potentially viable and require a further trigger to induce excystation. Each sample was counted in triplicate with 100 oocysts in each count and the viability was scored as shown in table 4.2.

### 4.2.3 Ozonation of Oocysts

A sample of approximately $1x10^6$ oocysts was prepared of each *C.parvum* isolate and suspended in 1ml of water prepared by reverse osmosis (RO) purification. The oocyst suspension was added to a Drechsel bottle containing 500ml of chilled RO water. Ozone gas ($O_3$) was produced by supplying a Hansler Lab II generator with an oxygen flow of 0.5 litres per min. The ozone supply in the water, which had been optimised to give a concentration of 1-2mg/ml, was connected when the water temperature reached 4.7°C. The ozone gas was initially bubbled into the bottle and through the water for 2min 30s to give maximum contact between oocyst and ozone. Any excess ozone was trapped in a second Drechsel bottle containing 0.4% (w/v) potassium iodide solution. The ozone supply was then disconnected and a 20ml sample of the water was collected to record the residual ozone concentration. The contact time was then extended to a total of 7min. At this point a second water sample was taken to measure the residual ozone concentration and the temperature was also recorded. Sodium thiosulphate (0.1N, 1ml) was added to the bottle to stop the reaction by removing any remaining ozone. The dissolved ozone concentration

**Table 4.2**

| Viability status | Type of Oocyst | Contents seen by Nomarski optics | Inclusion of DAPI | Inclusion of PI |
|---|---|---|---|---|
| (a) Viable | $DAPI^+/PI^-$ | Yes | Yes | No |
| (b) Viable after further trigger | $DAPI^-/PI^-$ | Yes | No | No |
| (c) Dead | $*(DAPI^+)/PI^-$ | Yes | *Yes | No |
| (d) Dead | $DAPI^+/PI^+$ | Yes | Yes | Yes |
| (e) Dead | Ghost | No | No | No |

* - Cytoplasmic DAPI staining- not solely nuclear staining, sporozoite membrane has ruptured.

**Table 4.2 Summary of *C.parvum* oocyst viability classification.** (a) $DAPI^+/PI^-$ - nuclei are positive for DAPI stain and negative for PI stain indicating that the nuclei are present and intact and the membrane is impermeable to PI. This signifies a viable oocyst. (b) $DAPI^-/PI^-$ - neither of the fluorogenic dyes have stained the sporozoite nuclei, although the presence of the nuclear contents has been confirmed by Nomarski optics. This signifies that the oocyst contains intact sporozoites, which may be viable following a further trigger enabling them to excise form the oocyst. These oocysts are classified as being potentially viable. (c) $*(DAPI^+)/PI^-$ - DAPI is present as a diffuse stain throughout the cytoplasm of the sporozoite. This indicates that the nuclear membrane has ruptured and the nuclear contents have leaked into the cytoplasm. The absence of the PI stain indicates that the outer sporozoite membrane remains intact and impermeable to the stain. These oocysts are non-viable due to the ruptured nucleus. (d) $DAPI^+/PI^+$ - both fluorogenic dyes have stained the nuclear contents of the sporozoites, indicating a non-viable oocysts. (e) Ghost - no nuclei have been stained and Nomarski optics has confirmed the absence of oocyst contents. These oocysts are considered non-viable.

was measured with the indigo trisulphonate method (International Ozone Association: Standardisation Committee-Europ 004/89 (F)). The ozonation of *C.parvum* was carried out with the expert assistance of Mr Ken Alexander, Ozotech Ltd, Burgess Hill, Sussex.

The oocysts were recovered from suspension by dividing the volume between two 250ml centrifuge bottles and centrifuging at 16000xg for 15min. The supernatant was aspirated to leave a volume of 3ml and the oocysts were then resuspended in this minimal volume. A 1ml sample was added to an Eppendorf tube and the oocysts were pelleted by centrifugation at 12500xg for 30s. The supernatant was aspirated to 20µl and a second 1ml sample was added. This process was repeated until all the oocysts were pelleted in the Eppendorf. The oocysts were resuspended in a minimal volume of 20µl and the volume was adjusted to 100µl. Oocysts were then examined for viability as described previously.

## 4.3 Results

The isolates selected for ozonation were chosen to provide a range of Type I and Type II oocysts. The Type II isolates where from a variety of animal hosts. A summary of the selected isolates is shown in Table 4.3. Each isolate was assayed for viability before and after ozonation. The viability data represented in Figure 4.2 was determined by combining the total of the viable ($DAPI^+/PI^-$) and the potentially viable ($DAPI^-/PI^-$) oocysts. This value is recognized as the percentage viability of the isolate. Ozonation appeared to have a range of effects on the viability of *C.parvum* oocysts. Some isolates appeared to show a significant reduction in viability whilst others remained apparently unaffected or showed an

**Table 4.3**

| *C.parvum* REFERENCE NO. | GENOTYPE | ORIGIN |
|---|---|---|
| C2 | II | *cervine/ovine |
| C3 | II | *cervine/ovine |
| 91019 | II | Bovine |
| 378 | I | Human |
| 317 | I | Human |
| 642 | II | Human |
| 633 | II | Human |
| 598 | II | Bovine |
| 652 | II | Bovine |

* - Isolate of cervine origin passaged through sheep and obtained from the Moredun Research Institute, Edinburgh, UK.

**Table 4.3 Summary of *C.parvum* isolates included in the ozonation experiment.** The genotype of the isolates was determined as indicated in section 4.2.2 of this thesis. The host origin of each isolate is also indicated.

increase in viability (Figure.4.2). These results suggest that the Human Type I isolates (317 and 378) were more susceptible to the ozonation treatment than the Type II isolates. It is important to note, however, that the viability of four Type II isolates C2, C3, 91019 and 642, appeared to increase following exposure to ozone and that the experiment was limited t a small number of isolates. A complete set of data is shown in tables 4.4 and 4.5.

The data was also analysed with respect to the individual components included in the overall percentage viability assessment; that is, the percentage of $DAPI^+/PI^-$ and $DAPI^-/PI^-$ oocysts before and after exposure to ozone (Figure 4.3). This figure clearly illustrates an increase in the proportion of potentially viable ($DAPI^-/PI^-$) oocysts following ozonation from that observed before exposure to ozone. Indeed, only one isolate, human Type I

isolate 378, had a greater percentage of viable oocysts than potentially viable oocysts following exposure to ozone.

**Figure4.2**

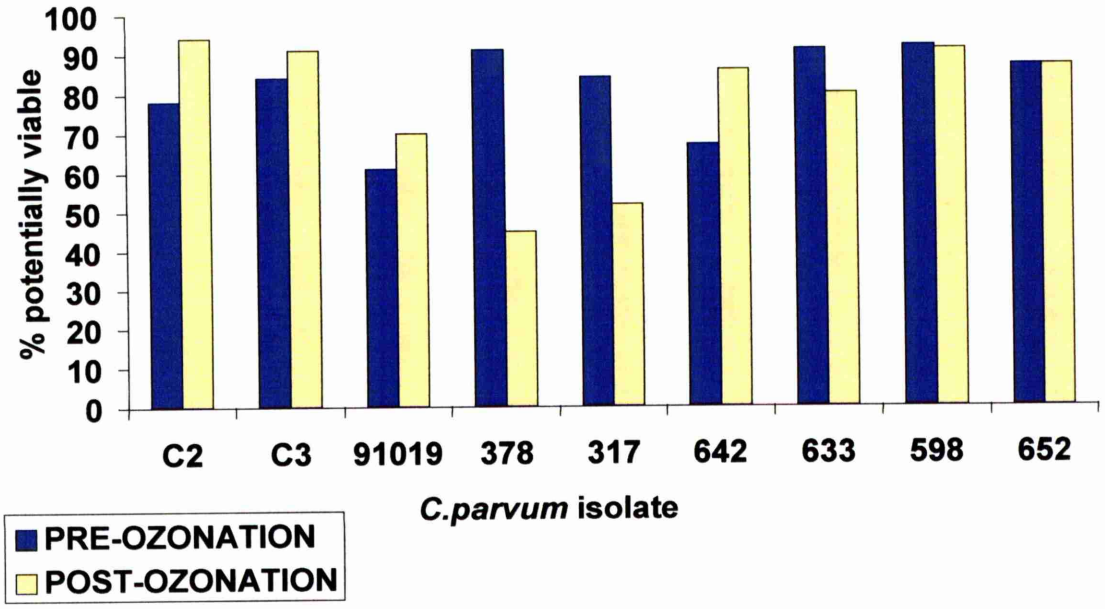


**Figure 4.2 *C.parvum* oocyst viability previous to and following exposure to ozone.** The viability of the oocysts was determined by the inclusion or exclusion of two fluorogenic vital dyes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) as described by Campbell *et al.* (1992). The percentage viability was determined as the sum of viable ($DAPI^+/PI^-$) and potentially viable ($DAPI^-/PI^-$) oocysts.

**Table 4.4**

| Sample | Type | Origin | Concentration ($x10^6$/ml) | $DAPI^+/PI^-$ | $DAPI^-/PI^-$ | $DAPI^-/PI^-$ | Ghosts | % Viable (Total $PI^-$) |
|---|---|---|---|---|---|---|---|---|
| C2 | II | C/O | 15 | 75 | 14 | 3 | 8 | 78 |
| C3 | II | C/O | 50 | 82 | 9 | 2 | 7 | 84 |
| 91019 | II | Bovine | 20 | 57 | 36 | 4 | 3 | 61 |
| 378 | I | Human | 27.7 | 91 | 9 | 0 | 0 | 91 |
| 317 | I | Human | 10 | 84 | 9 | 0 | 17 | 84 |
| 642 | II | Human | 11 | 67 | 29 | 0 | 4 | 67 |
| 633 | II | Human | 1.4 | 91 | 15 | 0 | 0 | 91 |
| 598 | II | Bovine | 1.6 | 91 | 7 | 1 | 1 | 92 |
| 652 | II | Bovine | 38 | 87 | 12 | 0 | 1 | 87 |

**Table 4.4 Details of the *C.parvum* isolates included in the ozonation study and the viability of each isolate determined before exposure to ozone.** This data was obtained from the viability assay performed on each isolate before exposure to ozone. The figures presented were calculated from the average of three counts, each of one hundred oocysts. Fully excysted oocysts, where the oocyst wall is present and the sporozoites have been released, were recorded as ghosts and are considered as non-viable. The percentage viability was calculated as the total of viable oocysts ($DAPI^+/PI^-$) and oocysts that are potentially viable following a further trigger ($DAPI^-/PI^-$).

**Table 4.5**

| Sample | Start Temp(°C) | End Temp(°C) | Residual $O_3$ (mg/l) (1) | Residual $O_3$ (mg/l) (2) | $DAPI^+/PI^-$ | $DAPI^+/PI^+$ | $DAPI^-/PI^-$ | Ghosts | %Viable (Total $PI^-$) |
|---|---|---|---|---|---|---|---|---|---|
| C2 | 4.7 | 6.4 | 1.10 | 1.07 | 15 | 6 | 79 | 0 | 94 |
| C3 | 4.7 | 6.3 | 1.15 | 1.18* | 1 | 9 | 90 | 0 | 91 |
| 91019 | 4.7 | 6.3 | 1.24 | 1.22 | 16 | 31 | 54 | 0 | 70 |
| 378 | 4.7 | 6.0 | 1.44 | 1.39 | 40 | 53 | 5 | 2 | 45 |
| 317 | 4.7 | 6.3 | 1.17 | 1.11 | 16 | 44 | 36 | 4 | 52 |
| 642 | 4.7 | 6.2 | 1.35 | 1.29 | 14 | 14 | 72 | 0 | 86 |
| 633 | 4.7 | 6.1 | 1.56 | 1.53 | 36 | 16 | 44 | 4 | 80 |
| 598 | 4.7 | 5.6 | 1.27 | 1.22 | 3 | 8 | 88 | 1 | 91 |
| 652 | 4.7 | 6.4 | 1.47 | 1.29 | 36 | 13 | 51 | 0 | 87 |

*-An unexplained increase in residual ozone concentration following disconnection of the ozone supply.

**Table 4.5 Parameters recorded during the ozonation study and the viability of each isolate determined after exposure to ozone.** The temperature of the water sample was recorded at the beginning and at the end of each ozonation reaction. (1) The ozone gas was bubbled through the water for 2min 30s. A sample was removed and the residual ozone concentration was measured as described in section 4.2.3. (2) Contact time was then extended to 7min, at which point the reaction was stopped by the addition of sodium thiosulphate and the final residual ozone concentration was recorded as before. The viability of the oocysts was determined as before. The percentage viability was calculated as indicated previously.

**Figure 4.3**

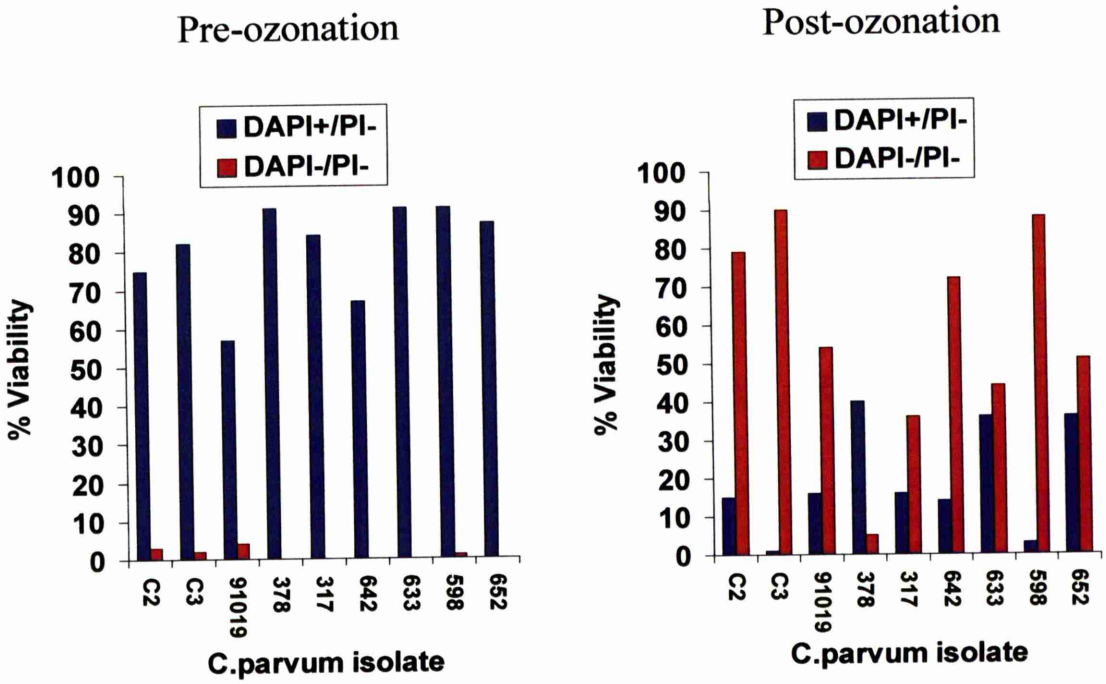


**Figure 4.3 The percentage of viable and potentially viable oocysts before and after exposure to ozone.** $DAPI^+/PI^-$ indicates that the oocyst has included DAPI stain while excluding PI, allowing them to be scored as viable at assay. Exclusion of both dyes

($DAPI^-/PI^-$) suggests that the oocysts is potentially viable following further trigger. The results show a dramatic increase in the percentage of $DAPI^-/PI^-$ oocysts following exposure to ozone and a significant decrease in the percentage of $DAPI^+/PI^-$ oocysts.

A significant example of the substantial increase in $DAPI^-/PI^-$ (potentially viable) oocysts is given in the cervine/ovine Type II isolate, C3. The overall viability of isolate C3 appeared to increase following ozonation (Figure 4.2); however, only 1% of these oocysts remained $DAPI^+/PI^-$, whilst $DAPI^-/PI^-$ oocysts increased from 2% pre-ozonation to 90% post-ozonation (Figure 4.3). The percentage of $DAPI^+/PI^-$ (viable oocysts) in isolate C3 decreased from 82% before ozonation to 1% following ozonation. Similarly, in the bovine Type II isolates 598 and 652 there is a marked

decrease in $DAPI^+/PI^-$ oocysts coupled with a distinct increase in the percentage of $DAPI^-/PI^-$ oocysts. As the overall viability in figure 4.2 accounted for both the viable and potentially viable oocysts, the viability appears to have increased by exposure to ozone.

The significance of the $DAPI^-/PI^-$ oocysts is highlighted in figure 4.4, which illustrates the percentage of non-viable oocysts ($DAPI^+/PI^+$) before and after exposure to ozone. When the percentage of $DAPI^+/PI^+$ oocysts were examined before and after exposure to ozone, only in isolates 317 and 378 (Human Type I) was there evidence of and increase in non-viable oocysts.

**Figure 4.4**

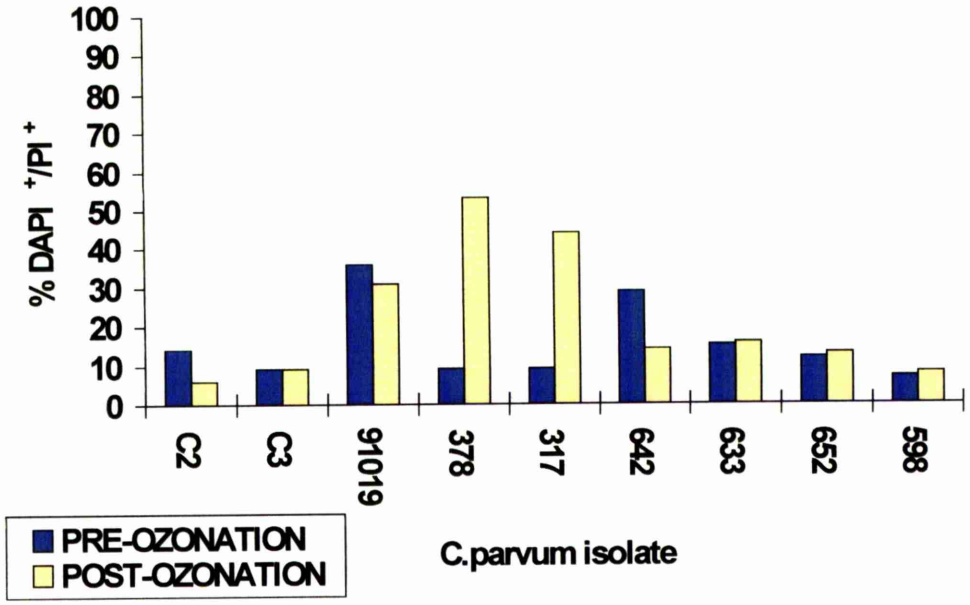


**Figure 4.4 The percentage of non-viable oocysts before and after exposure to ozone.** The correlation of the results shown here with the $DAPI^+/PI^-$ viable oocyst results shown in Figure 4.3 highlights the influence of the potentially viable oocysts on the overall viability of each isolate displayed in Figure 4.2.

## 4.4 Discussion

Ozone inactivation of *C.parvum* oocysts was performed on a panel of nine different isolates of known genotype and from a range of human and animal hosts. The viability of the oocysts was determined by a single assay involving the differential uptake of two fluorogenic vital dyes, DAPI and PI.

This study demonstrates that ozonation did have an effct on *C.aprvum* oocysts, but the significance of the results are less clear. In both Human Type I isolates ozonation appeared to have an unambiguous effect on viability. However, in all other Type II isolates the most common occurance was dramatic shift from viable ($DAPI^+/PI^-$) to potentially viable ($DAPI^-/PI^-$) oocysts. Since morphological disruption was also common, the true infectivity of these potentially viable oocysts is not clear. The morphological disruption could be investigated by considering the structure and components of the layered oocyst wall (Harris & Petry, 1999).

Previous studies employing different viability methods have resulted in conflicting observations. Campbell *et al.* (1992) reported a strong correlation between the vital dye assay and an *in vitro* excystation assay. They found that an increase in $DAPI^-/PI^-$ oocysts resulted in a decrease in excystation and, hence, viability. There were no animal infectivity experiments performed at that point to uphold these results. However, Jenkins *et al.* (1997) reported the opposite, where $DAPI^-/PI^-$ oocysts were able to excyst and infect mice. They suggested that the fluorogenic vital dye assay gave a conservative estimate of isolate viability and potential infectivity. Furthermore, Jenkins *et al.* (1997) reported that the vital dye assay indicated only whether the oocyst wall was permeable and not the metabolic activity (or infectivity)

of the sporozoites. It has also been suggested that the age, storage conditions and cleaning methods used for each isolate may influence the permeability of the oocyst wall and, therefore the results of the vital dye assay (Gasser & O'Donoghue, 1999).

Campbell *et al.*, (1992) showed that the number of $DAPI^-/PI^-$ oocysts could be reduced by an acidification step prior to incubation with the vital dyes. This step was intended to simulate the environment experienced by the oocysts following ingestion and was included in this assay to ensure optimal assay conditions. It is possible that the greatly increased number of $DAPI^-/PI^-$ oocysts after exposure to ozone, which was more pronounced in the Type II isolates, may have been due to disruption to the structure of the oocyst wall. Many oocysts displayed severe morphological disruption as a result of the ozonation procedure. This disruption may also have induced a change in the oocyst wall structure, which allowed for the retention of the sporozoite contents while preventing the uptake of the vital dyes. Although the appearance of such oocysts suggests that they remain potentially viable, it is possible that these oocysts would be unable to release their sporozoite contents due to the distinct morphological disruption to their oocyst wall. The viability assay employed in this study does not provide enough evidence to draw any strong conclusions on this point. Nevertheless, it is important to recognise that recording $DAPI^-/PI^-$ oocysts as potentially viable without further verification might result in an overestimation of overall viability following ozonation.

Previous studies conducted on ozone inactivation of *C.parvum* oocysts have also reported discrepancies between the viability assays employed. Rennecker *et al* (1999) compared two different excystation assays and observed inconsistency between them. This group also observed some unexpected results, as reported here,

where they believed that certain oocysts might have been wrongly classified due to their ambiguous appearance following ozonation. Further studies by Finch *et al.* (1993) compared *in vitro* excystation with animal infectivity of oocysts following ozonation. This group discuss an underestimation of oocyst inactivation by the *in vitro* excystation assay they employed in comparison with the animal infectivity experiments that they had also completed and they conclude that animal infectivity is the best option for viability determination. This observation and opinion was echoed by Bukhari *et al* (2000) who concluded that the three vital-dye-based assays and *in vitro* excystation assay that they tested gave a significant overestimation of viability following ozonation when compared with animal infectivity experiments that they also performed. A consensus seems now to be that animal infectivity experiments are the best method currently available for determining oocyst viability.

Nevertheless, the observations from this study suggest that there appears to be variation in susceptibility to ozonation between *C.parvum* isolates although the sample number was small and further studies are required. However, the viability assay used in this study is limited to giving an indication of the potential viability of the oocysts and not actual viability. Further analysis such as an excystation assay or animal infectivity experiments may prove more useful in confirming our data.

If further studies do confirm our observations of variation in susceptibility between *C.parvum* isolates when exposed to ozone, this would have potentially important implications for the purification of water supplies by ozone as it may imply that viable *C.parvum* oocysts could remain present in the water. Further study is required to investigate the mechanism by which ozone disrupts the parasite and to what extent they become inactivated.

## Chapter Five: Discussion

Currently, the control of cryptosporidiosis relies on limiting the transmission of *C.parvum*, due to the lack of effective treatment for the infection. Ozone has been investigated as a potential disinfectant for the inactivation of *C.parvum* oocysts in water supplies, which is a common root of contamination and cause of infection. Previous studies reported a variation in susceptibility to ozone exposure (Parker, 1993, Parker *et al*, 1993, Rennecker, *et al*, 1999). One possible explanation for this observation could be that *C.parvum* oocysts are able to provide different degrees of protection against ozone. This protection is likely to be provided by antioxidant enzymes produced by the parasite for defense against native reactive oxygen species resulting from aerobic metabolism. Further studies reported on the antioxidant enzymes present in *C.parvum* and this suggested that *superoxide dismutase* was present while all other antioxidant enzymes investigated were not detected (Entrala, *et al*, 1997). From these observations stemmed the initial concept to clone and characterise a *SOD* gene from *Cryptosporidium parvum.*

An internal fragment of a *SOD* gene from *C.parvum* was identified from genomic DNA. The sequence displayed high homology with *SOD* gene sequences from a number of other organisms, most notably *P.falciparum*. From comparison with other *SOD* sequences, it was estimated that there were approximately 80bp at either end of the gene remaining to be identified.

Sequence analysis of this internal fragment of the gene revealed details of the SOD protein. The biochemical observations reported by Entrala *et al*, (1997), suggested that a Fe-dependant SOD was present in *C.parvum* and the molecular analysis presented here appears to support this. Conserved sites that are distinct between the three types of SOD: Mn-SOD, Cu/Zn-SOD and Fe-SOD, were considered to characterise the isolated sequence. The presence of a single Fe-SOD is common among parasitic protozoa and this is unlike the situation found in mammals, which commonly produce both Mn-SOD and Cu/Zn-SOD. The distinction between the form of SOD found in *C.parvum* from that present in the mammalian host has highlighted the possibility of the Fe-SOD as a target for drug therapy. The issue of potential drug targets of *C.parvum* has been reviewed by Coombs, (1999), and specific consideration of the *SOD* gene as a drug target has been suggested n related organisms (Baert, *et al*, 1999(a), Baert, *et al*, 1999(b), Krauth-Siegel & Coombs, 1999).

The preliminary data on the *SOD* gene of *C.parvum* has provided a base for further investigation into the antioxidant enzymes of the parasite. A recent genome wide survey has identified a putative glutathione reductase homologue (Strong & Nelson, 2000), which they suggest could be a suitable drug target, although this contradicts the biochemical data from Entrala *et al*, (1997), in which they failed to detect glutathione reductase activity. *C.parvum* does appear to show peculiarities in relation to other protozoa, however other antioxidant enzymes have been identified and characterised in related parasites and it is possible that they are also present in *C.parvum*.

The suggestion of a Fe-dependent *SOD* from molecular analysis could be extended to encompass protein analysis. Inhibition assays have been established to indicate each form

of SOD according to the susceptibility to certain compounds: Cu/Zn-SODs are sensitive to cyanide and $H_2O_2$, Mn-SODs are resistant to both cyanide and $H_2O_2$, while Fe-SODs are resistant to cyanide and sensitive to $H_2O_2$. Further information would be obtained by determining the cellular location of the SOD protein.

The *SOD* gene of *C.parvum* has been shown to contain polymorphic nucleotides suitable for differentiating between Type I and Type II genotypes. The application of genotyping *C.parvum* isolates could be enhanced by simple analysis of the products of PCR amplification of the marker genes. This could be achieved by a simple restriction endonuclease digest that distinguishes between isolates, as has been reported by Spano *et al*, (1997). They identified a polymorphism within the COWP gene that altered the recognition site of the endonuclease *Rsa*I and this enzyme could be used for rapid differentiation between PCR products amplified from the COWP marker gene. It is theoretically possible that this could be also applied to the *SOD* gene, as the polymorphism at position 361 of the internal fragment of the gene alters the recognition sequence of two restriction endonucleases: *Bsp*HI and *Rca*I. Both enzymes recognise the same palindromic sequence 5' tcatga 3', which is present in the *SOD* sequence of Type II oocysts but differs in Type I isolates by a thymidine to cytosine substitution in the fourth position. This would be an interesting hypothesis to test and could be incorporated to provide a simple tool for rapid differentiation between *C.parvum* genotypes.

The identification of polymorphic sites within the *C.parvum SOD* sequence may prove useful as a genetic marker but it is unlikely that these polymorphisms will have a direct effect on the protein encoded by the gene. All seven polymorphisms are present in the

third position of each codon, which has prevented any alteration to the secondary structure of the gene. It is also evident that the polymorphisms are not present in highly conserved regions of the gene and, therefore, are unlikely to interfere with the catalytic ability of the enzyme. It is also interesting to note that each polymorphism is the result of the substitution of a pyrimidine for another pyrimidine or a purine for another purine. The significance of this observation is not clear but could simply be due to the spatial arrangement of the nucleotides within the DNA molecule, as complementary nucleotides retain the structural and size limitations of the DNA double helix.

It has been suggested that *C.parvum* may exist in a clonal population, due to an apparent lack of genetic recombination between the different genotype (Awad-El-Kariem, 1999). This appears to be the case, even though oocysts of both genotypes can co-exist in a single host. However, recent experimental data has been presented which contradicts previous understanding of this issue (Feng, *et al*, 2002) and suggests that sexual recombination can occur between *C.parvum* genotypes and, therefore, may also occur in nature.

The exposure of *C.parvum* oocysts to ozone has been included in numerous studies for water purification techniques. Although different studies have supported the idea of ozone as a suitable reagent for oocyst inactivation, the levels of ozone required have proven to be high for significant levels of inactivation (Rennecker, *et al*, 1999, Korich, *et al*, 1990, Finch, *et al*, 1993). There are specific guidelines in place to regulate the bi-products of reagents employed in purification techniques, which limits the levels of detergents that can be safely applied. The methods used to test oocysts viability have varied and, therefore, results have been inconsistent and occasionally contradictory. The results of the ozone

inactivation study presented here have confirmed previous observations of differential susceptibility to ozone, although, there is no clear correlation of this variation with isolate genotype. However, these results are sufficient to suggest that *C.parvum* oocysts behave differently when exposed to ozone and this merits further investigation. Recent studies have involved the use of multiple detergents to establish a protocol for enhanced inactivation of oocysts with acceptable application parameters for drinking water and recreational water supplies (Li, *et al*, 2001, Corona-Vasquez, *et al,* 2002).

The future of *Cryptosporidium parvum* genomic studies is set to change with the completion of the genome sequence. Gene cloning could be achieved entirely *in silico* and the construction of a *C.parvum* micorarray chip would allow for genome wide analysis of gene expression and sequence identification. The development of an effective *in vitro* cultivation technique for *C.parvum* will enable such advancements and would open the door for more extensive investigations into the behaviour of the parasite. For example, characterisation of putative gene sequences could be established by the construction of gene knockout clones, although, the lack of an efficient *in vitro* cultivation system and transfection method for *C.parvum* could prevent such an achievement. Other options may include RNA interference experiments to silence the *SOD* gene, which, in the future could enable the study of the effects of ozone exposure on *SOD*–defficient clones to give a clear indication of the protection provided by the only antioxidant enzyme identified from *C.parvum*, to date.

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