DETERMINATION OF IgG ANTIBODY LEVELS TO
CRYPTOSPORIDIUM PARVUM OOCYST ANTIGENS IN
VERVET MONKEYS (CERCOPITHECUS AETHIOPS)
INFECTED WITH VARIOUS DOSES OF HUMAN-DERIVED
C. PARVUM OOCYSTS.

DRAFT FINAL REPORT TO THE DEPARTMENT OF THE ENVIRONMENT IN
FULFILMENT OF WRe CONTRACT NUMBER ESL 9517 AWARDED TO:

Scottish Parasite Diagnostic Laboratory (SPDL),
Stobhill Hospital NHS Trust, Glasgow G21 3UW, Scotland.

in conjunction with the

Kenya Trypanosomiasis Research Institute (KETRI),
PO Box 362, Kikuyu, Kenya.

***

Smith H.V., Sayer P.D., Bukhari, Z., Ngotho J.M.,
Ndung'u J.M, Ngure R.M. and Grimason A.M.

***

July 1994

CRYPTOSPORIDIUM IN WATER
DoE Reference: PECD 7/7/357

Subcontractors Report CR 15
STATEMENT OF POSITION
The serological study described herein completes the work identified in WRc contract number ESL 9517. It should be read in conjunction with the draft final report to the Department of the Environment entitled “Determination of the minimum infective dose of Cryptosporidium parvum oocysts in vervet monkeys (Cercopithecus aethiops)”.

INTRODUCTION
In partial fulfilment of WRc contract number ESL 9517, Cryptosporidium oocysts derived from symptomatic human cases were administered orally to adult, juvenile and infant vervet monkeys (Cercopithecus aethiops) at doses of either $1 \times 10^5$ or $1 \times 10^4$ per os. None of the animals infected developed discernible signs of cryptosporidiosis during a follow up period of four to six weeks. Similarly no oocysts were detected in the faeces of any animal during a follow up period of four to six weeks. In this study, the possibility of innate resistance to infection either at a specific (immune) or non-specific (phenotypic) level could not be excluded. In an attempt to determine whether specific immunity played a role in this animal model, sera from all the animals dosed were assayed for the presence of IgG isotype-specific antibodies to \textit{C. parvum} oocyst antigens by the enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

\textit{Preparation of antigen}

Sporulated cervine-ovine Cryptosporidium parvum oocysts, with a viability of 80\%, were obtained from the Moredun Research Institute, Edinburgh.

A water-soluble antigen extract of sporulated \textit{C. parvum} oocysts was prepared by subjecting $10^7$ oocysts suspended in phosphate buffered saline (PBS, 150 mM, pH 7.2) to a freeze-thawing regime. The protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF) was added to the oocyst suspension to a concentration of 10 mM in order to prevent breakdown of proteinaceous antigens. The oocyst suspension was immersed in liquid nitrogen until frozen and transferred to a waterbath at 37°C until thawed completely. This procedure was repeated a further two times. The extract was centrifuged at 13,000 x g for 30 min to remove particulate material, and the supernatant was aliquoted and stored at -20°C until used.

\textit{ELISA procedure}

Antigen was diluted optimally (1:500) in carbonate-bicarbonate buffer, pH 9.6, and 50 \mu l aliquots coated onto individual wells of 96-well microtitre plates (Dynatech M129B) for 2 h at 37°C. The plates were rinsed in PBS containing 0.05\% Tween 20 (PBST) to remove unbound antigen, air dried and stored at -20°C for up to a week. Chequerboard titrations were performed for all the reagents used in order to identify their optimal working dilutions. Monkey sera were stored at -20°C at KETRI, transported to the SPDL in frozen CO₂ and stored at -20°C at the SPDL until used. Test and control sera were diluted to 1:100 in PBST containing 2\% bovine serum albumin prior to use.

Aliquots (50 \mu l) of previously diluted monkey test sera were placed in the wells of antigen-coated plates, following which the plates were sealed and shaken for 5 min at room temperature and then incubated without shaking for 1 h at 37°C. Plates were washed x3 in PBST and tapped dry. Fifty 50 \mu l aliquots of anti-monkey IgG (Nordic Immunological Laboratories, Ltd., raised in goat, and diluted optimally to 1:1600) were added to each well, following which the plates were sealed and shaken for 5 min at room temperature and then incubated without shaking for 1 h at 37°C. Plates were washed x3 in PBST and tapped dry.

Fifty 50 \mu l aliquots of anti-goat IgG conjugated to horseradish peroxidase (Scottish Antibody Production Unit, raised in rabbits, and diluted optimally to 1:3200) were added to each well, following which the plates were sealed and shaken for 5 min at room temperature and then
incubated without shaking for 1 h at 37°C. Plates were then washed 3x in PBS1 and tapped dry. Fifty 50 μl aliquots of a solution containing H₂O₂ and tetramethyl benzidine (Kirkegaard and Perry Laboratories, Inc.) were added to each well, incubated for 5 min, and the optical density (OD) of the colour developed was read at 450 nm on a Titertek Plus ELISA reader. Known negative and positive controls were included in every assay.

**Sera**
In the absence of known negative and positive vervet monkey sera, the following sera were used to determine the sensitivity and specificity of the ELISA.

a) Sera from mice infected/immunised with cervine-ovine-derived oocysts of *C. parvum*. These were used as positive control sera in the ELISA.

b) Uninfected vervet monkey and human cord sera. These were used as negative control sera in the ELISA.

**RESULTS**
Using this assay format, the mean background OD (without control or test sera) was 0.044 (range 0.042 to 0.047, n = 15) and the mean OD for negative sera (1:100 dilution) was 0.22 (range 0.137 to 0.352, n = 26). Sera from mice infected/immunised with cervine-ovine-derived oocysts of *C. parvum* consistently produced OD readings of >1.8 units.

The minimum (threshold) OD value for positivity was set at the mean OD for negative sera PLUS three times the standard deviation of the mean (mean = 0.22 ± 0.053; 3 x s.d = 0.159).

Thus any monkey serum samples with an OD below 0.4 could be regarded as lacking an IgG response.

The results of the ELISA for the demonstration of IgG antibodies to *C. parvum* oocyst antigens are presented in the accompanying figures. The tables of the various experimental regimes are included for completeness.

**Adult vervet monkeys**
On three occasions, values for the negative control (122) were above the threshold for positivity (06/03/91, 21/03/91, 04/04/91).

Serum samples analysed prior to infection failed to reveal evidence of IgG antibodies to oocyst antigens. Antibody levels in post-infection serum samples fluctuated around the threshold level for much of the duration of the experiment, and in some instances positive values were recorded (number 88: 31 [28/03/91] and 38 [04/04/91] days post infection; number 94: 24 [21/03/91], 31 [28/03/91] and 28 [04/04/91] days post infection; number 91: 17 [14/03/91] days post infection; number 117: 17 [14/03/91], 24 [21/03/91], 31 [28/03/91], 38 [04/04/91] and 42 days post infection, (Trial I, Figures 1a and 1b).

In those adults infected experimentally with either 10⁴ or 10⁵ oocysts and which sero-converted, a transient increase in OD values followed by a decline in values towards the end of the follow-up period was observed (88, 91, 94, 117) (Trial I, Figures 1a and 1b).

**Juvenile vervet monkeys**
Serum samples analysed prior to infection failed to reveal evidence of IgG antibodies to oocyst antigens. Antibody levels in post-infection serum samples were detected in three of the four experimental animals (262, 263, 264). In these animals, antibody OD values rose gradually during the experiment and appeared to tail off at the end of the follow-up period (Trial II, Figures 2a and 2b).

**Infant vervet monkeys**
Serum samples analysed both prior to and following infection failed to reveal evidence of IgG antibodies to oocyst antigens (Trial III, Figure 3).
### Trial I: Adults (wild caught)

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Age in years</th>
<th>No. of Oocysts administered</th>
<th>Follow-up period</th>
<th>Oocyst output in faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>male</td>
<td>&gt;5</td>
<td>$1 \times 10^4$</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>88</td>
<td>male</td>
<td>&gt;5</td>
<td>$1 \times 10^4$</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>94</td>
<td>female</td>
<td>&gt;5</td>
<td>$1 \times 10^4$</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>117</td>
<td>female</td>
<td>&gt;5</td>
<td>$1 \times 10^5$</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>124</td>
<td>male</td>
<td>&gt;5</td>
<td>$1 \times 10^5$</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>91</td>
<td>male</td>
<td>&gt;5</td>
<td>Nil</td>
<td>42 days</td>
<td>Nil</td>
</tr>
<tr>
<td>122</td>
<td>female</td>
<td>&gt;5</td>
<td>Nil</td>
<td>42 days</td>
<td>Nil</td>
</tr>
</tbody>
</table>

### Figures 1a and b.

**IgG antibody response to *C. parvum* oocyst antigens in adult monkeys dosed with $10^4$ oocysts**

![Graph 1a](image)

**IgG antibody response to *C. parvum* oocyst antigens in adult monkeys dosed with $10^5$ oocysts**

![Graph 1b](image)
Trial II: Juveniles (wild caught)

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Age</th>
<th>No. of oocysts administered</th>
<th>Follow-up period</th>
<th>Oocyst output in faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>262</td>
<td>female</td>
<td>sub-adult</td>
<td>1.5x10^4</td>
<td>28 days</td>
<td>Nil</td>
</tr>
<tr>
<td>263</td>
<td>male</td>
<td>sub-adult</td>
<td>1.5x10^4</td>
<td>28 days</td>
<td>Nil</td>
</tr>
<tr>
<td>264</td>
<td>male</td>
<td>sub-adult</td>
<td>1x10^5</td>
<td>28 days</td>
<td>Nil</td>
</tr>
<tr>
<td>265</td>
<td>female</td>
<td>sub-adult</td>
<td>1x10^5</td>
<td>28 days</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Figures 2a and b.

IgG antibody response to *C. parvum* oocyst antigens in juvenile vervet monkeys dosed with 1.5 x 10^4 oocysts

![Graph showing IgG antibody response to *C. parvum* oocyst antigens in juvenile vervet monkeys dosed with 1.5 x 10^4 oocysts]

IgG antibody response to *C. parvum* oocyst antigens in juvenile vervet monkeys dosed with 10^5 oocysts

![Graph showing IgG antibody response to *C. parvum* oocyst antigens in juvenile vervet monkeys dosed with 10^5 oocysts]
### Trial III: Infants (captive bred except No. 369)

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Age in months</th>
<th>No. of oocysts administered</th>
<th>Follow-up period</th>
<th>Oocyst output in faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>369</td>
<td>male</td>
<td>8, weaned</td>
<td>$1 \times 10^5$</td>
<td>30 days</td>
<td>Nil</td>
</tr>
<tr>
<td>371</td>
<td>male</td>
<td>7, unweaned</td>
<td>$1 \times 10^5$</td>
<td>30 days</td>
<td>Nil</td>
</tr>
<tr>
<td>373</td>
<td>male</td>
<td>6, unweaned</td>
<td>$1 \times 10^5$</td>
<td>30 days</td>
<td>Nil</td>
</tr>
<tr>
<td>375</td>
<td>male</td>
<td>3, unweaned</td>
<td>$1 \times 10^5$</td>
<td>30 days</td>
<td>Nil</td>
</tr>
<tr>
<td>377</td>
<td>male</td>
<td>6, unweaned</td>
<td>$1 \times 10^5$</td>
<td>30 days</td>
<td>Nil</td>
</tr>
</tbody>
</table>

**Figure 3.**

IgG antibody response to *C. parvum* oocyst antigens in infant monkeys dosed with $10^8$ oocysts

![Graph showing antibody response]
DISCUSSION

Vervet monkeys inoculated with oocysts of human origin were bled at prescribed times before and during these trials. Serum samples were collected as indicated and analysed for the presence of IgG by an indirect ELISA developed at the SPDL. Whilst only one control serum sample was included in the series identified in this report, examination of pre-infection bleeds from animals infected with C. parvum oocysts by ELISA failed to demonstrate the presence of IgG antibodies to C. parvum oocyst-specific antigens. In addition, analysis of sera from other uninfected vervet monkeys, held at KETRI during this infectious dose trial, failed to demonstrate levels of antibody above the cut-off level for positivity (>0.4 OD units, data not shown).

Sera from the majority of adult and juvenile monkeys infected with oocysts demonstrated increased IgG antibody values, above the cut-off level for positivity at some time during the trials (Figures 1a and b, 2a and b), and this increase in IgG antibody is indicative of exposure to sporulated oocyst antigens. We feel that it is unlikely that the antibody response was due to sensitisation during passage of the oocyst inoculum through the gastro-intestinal tract because of the duration of the heightened antibody response. Furthermore, the decline in OD values towards the end of the follow-up period is suggestive of the waning of a response to a stimulus encountered over a period of time. Finally, analysis of the trends in antibody OD values between adults infected with either $10^4$ or $10^5$ identifies a more rapid increase in IgG antibody positivity in the animals infected with the higher infectious dose. The most likely explanation for these serological data is as a result of the sporozoites in the experimental inoculum excysting in vivo and producing either a transient or low level Cryptosporidium infection in these antibody positive animals.

Whilst IgG antibody OD values rose gradually and appeared to tail off at the end of the follow-up period in juvenile vervet monkeys (Trial II), no serological evidence of IgG antibodies to oocyst antigens was obtained from the sera of infant vervet monkeys (Trial III). Again, the slow increase in antibody positivity in juvenile sera is suggestive of a primary rather than an anamnestic IgG response to the antigens tested, whilst the lack of an identifiable IgG response in sera from infants is suggestive of these animals being immunologically naive to these antigens. The data presented do not argue for a solid immunity to C. parvum, which would prevent the establishment of infection, in the animals tested. Without doubt, investigations into the presence of other antibody isotypes, especially IgM and IgA, and the production of cytokines during infection would help unravel this puzzle.

When the parasitological and serological arguments are combined it seems likely that these vervet monkeys were infected, but produced little in the form of either oocysts or IgG antibody. An explanation for the induction of a transient or low level infection occurring in vervet monkeys may be that these animals are not as susceptible to human-derived C. parvum oocysts as were our human donors. We suggested the possibility of strain or intra-species variation within C. parvum in our previous report (WRC contract number ESL 9517). Recently, evidence for intra-species variation in C. parvum oocysts isolated from different hosts has been forthcoming. Utilising techniques such as DNA restriction fragment length polymorphism (Ortega et al., 1991), Western blotting with polyclonal and monoclonal antibodies (Nina et al., 1992) and iso-enzyme typing, differences between isolates of C. parvum oocysts have been demonstrated.

Furthermore, cross-transmission studies of oocysts isolated from AIDS patients with mild cryptosporidiosis have resulted in severe disease in neonatal calves, whereas oocysts isolated
from AIDS patients with severe cryptosporidiosis only produced mild disease in neonatal calves (Pozio et al., 1992). Thus, the possibility exists that the oocysts of human origin (derived from a symptomatic individual), which were utilised for the infectivity trials, may not have been particularly virulent for vervet monkeys.

In addition to intra-species variation in *C. parvum*, non-specific resistance to colonisation by *C. parvum* has also been demonstrated since the completion of these trials. Resistance has been demonstrated in SCID mice (lacking B- and T-cell function), where its expression was demonstrated to be dependant upon the presence or absence of intestinal flora (Harp et al., 1992). Additionally, parameters such as age-related resistance (Novak and Sterling, 1991, Harp et al., 1990) of the host and resistance at a genetic level (Rasmussen and Healey, 1992) have also been documented in Cryptosporidium infections. These data strengthen our previous arguments for the possibility of innate resistance to infection either at a specific (immune) or non-specific (phenotypic) level occurring in this non-human primate model. It is unfortunate that such data were not available when these trials were instigated.

**CONCLUSIONS**

1. Whilst parasitological evidence based on the analysis of faeces for oocysts failed to provide evidence of infection in the vervet monkeys infected with *C. parvum* oocysts, some serological evidence is available which suggests that these animals became infected, albeit either transiently or with a low grade of infection.

2. Oocysts of human origin (in this instance derived from a symptomatic individual), used in these trials, may not have been particularly virulent for vervet monkeys.

3. The possibility of innate resistance to infection at a non-specific (phenotypic) level cannot be excluded. The possibility of resistance to infection at a specific (immune) level could be investigated further.

4. Since the completion of this contract, evidence for strain or intra-species variation within *C. parvum* has been forthcoming. As recommended previously, the effects of such variation should be given sufficient consideration.
REFERENCES


