Haematological and Serological Findings in *Cercopithecus aethiops* (African green monkeys) with *Cyclospora* Infections in Kenya

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Abstract

Cyclospora,is a common cause of gastroenteritis in humans resulting in protracted life threatening diarrhoea in immunocompromised patients worldwide.*Cyclospora* infections have been reported in African green monkeys. The objective of the study was to determine the haematological and serological parameters in African green monkeys with *Cyclospora* infections.A cross-sectional laboratory based study was done at Institute of Primate Research, Nairobi, from March 2008 to June 2009. Thirty three African green monkeys comprising of 10 male adults, 7 female adults, 3 male juveniles and 13 female juveniles were analysed for blood cell counts using a haematological analyser and screened for *Cyclospora* by ELISA. The red blood cell counts decreased in all study animals with mean decline of 1.2-1.5 (x 10^6/µl). The white blood cell counts varied among the study animals with highest increase among female adults from 4.3-7.1 to 5.4-8.8 (x 10^3 /µl) and a decrease among the male adults from 3.9-8.7 to 1.5-5.3 (x 10^3/µl). Positive antibody responses to *Cyclospora* were observed in 20 of the study animals with highest mean optical density (OD) values of 0.816 ± 0.100 observed among the female juveniles and lowest among the male juveniles at mean OD value of 0.646 ± 0.055. In conclusion, *Cyclospora* infections triggered both cellular and humoral responses in the African green monkeys.

Key words:*Cyclospora, Juvenile,*

IJPP 2013, 4(1&2): 122-131

Introduction

Cyclosporacayetanensis is emerging protozoan parasite causing diarrhoea in immunocompetent and immunocompromised patients worldwide (Ortega *et al.,* 1994; Wurtz, 1994; Bern *et al.*, 1999; Nassef *et al.*, 1998; Ceqielski *et al.*, 1999; Eberhard *et al.*, 1999; Fryauff *et al.*, 1999; Bern *et al.*, 2002; Kumar *et al.,* 2002; Alakpa *et al.*, 2003). In immunocompetent individuals the disease is usually self-limiting but in the immunocompromised, particularly acquired immunodeficiency syndrome (AIDS) patients, *Cyclospora* may cause persistent diarrhoea (Berlin *et al.*, 1994; Sifuentes-Osornio *et al.*, 1995; Goodgame, 1996; Soave, 1996; Fleming, 1998; Mohandas *et al.,* 2002; Turk *et al.*, 2004).

One of the major health problems among HIV seropositive patients is superimposed infections due to the defect of immunity. Intestinal parasite infections are common in tropical countries and have high prevalence among HIV-infected patients (Wiwanitkit, 2001). Coccidian protozoa *C. cayetanensis*, *Cryptosporidium spp* and *Isospora belli*, which are opportunistic infections, are all causes of chronic severe diarrhoeal diseases in the context of HIV interactions. Several studies have recorded co-infections of *Cyclospora* with HIV (Pape *et al.*, 1994; Sifuentes-Osornio *et al.*, 1995; Verdier *et al.*, 2000).

Asymptomatic *Cyclospora* infections have been reported in non-human primates from Tanzania, Ethiopia, Kenya and Sri Lanka (Smith *et al.*, 1996; Legesse and Erko, 2004; Eberhard *et al.,* 2001; Ekanayake *et al.*, 2006). The infections are widespread in non-human primates with prevalence ranging from 9 to 35% (Eberhard *et al.*, 2000; Legesse and Erko, 2004). The objective of the current study was to determine the haematological and antibody responses of *Cercopithecus aethiops* with *Cyclospora* infections.

Materials and Methods

Study area and study animals

The study animals were wild-trapped from Lemuria Forest Reserve, Aberdare; Arboretum Forest, Nairobi andNguruman in Kajiado district and transported to Institute of Primate Research (IPR), Nairobi, Kenya. Two female juveniles were colony bred at IPR. Thirty three *Cercopithecus aethiops* (African green monkeys) comprising of 10 male and 7 female adults, 3 male and 13 female juveniles were used in the study. None of the animals had symptoms at the time of sampling.

Faecal sampling, processing and Cyclospora identification

Four faecal samples per individual animal were collected on Mondays and Thursdays for two weeks and processed by standard formal ethyl acetate floatation technique. Faecal smears were prepared from the sediments, stained with hot safranin and examined by light microscopy for *Cyclospora*oocysts.

Blood sample collection, processing and haematology

Animals were immobilized with 10mg/kg bwt Ketamine (Ketasetœ, Fort Dody Iowa) plus 6 mg/kg bwt Xylazine (Chanazine, Ireland) at the ratio of 5:3 for sampling. A blood sample (5ml) was collected through venepuncture of the femoral vein from each

study animal. A 3 ml blood sample per individual animal was used to prepare serum for *Cyclospora* specific responses assay by antibody ELISA. A 2mlsample of heparinised blood by individual study animal was analysed for complete blood cell count using haematological analyser (Coulter counter, Beckman Coulter, Miami, USA).

Cyclospora soluble protein preparation

Cyclospora oocysts harvested from positive monkeys by modified salt floatation technique were disrupted by probe sonication for 10X for 1 minute with rest intervals using sonicator (Soniprep 150) at 25 watts. The BIO-RAD method (BioRad, 1976) was used to quantify the amount protein of protein in the mixture Bovine serum albumin {(BSA) (Sigma Chemical Company, USA)} as a standard and the test protein had a concentration of 1mg/ml.

Cyclospora Specific Enzyme linked Immunosorbent Assay

The protein was diluted to10µg/ml in bicarbonate buffer at pH 9.6 and a volume of 100µl of diluted protein was used to coat a 96 well microtitre ELISA plate (Nunc Roskilde, Denmark) and incubated over night at +4^{0}C. Further reaction was blocked with 3% BSA in phosphate buffered saline (PBS) and the plates were washed with 0.05% Tween 20 in PBS using ELISA plate washer. The test and control serum harvested as described above were diluted 1:100 in 1% BSA PBS-Tween 20 solution (dilution buffer). A 100µl Human anti-monkey IgG conjugated to horse radish peroxidase (Sigma Immunochemicals Mo, USA) diluted to 1:2000 in dilution buffer was added to each well. The plates were incubated for 1 h at 37^{0}C. A volume of 100µl TMB microwell peroxidase substrate (SureBlue™, Garthersburg, USA) was added to each well and the plates incubated for 30 minutes at ambient temperature in the dark. The reaction was stopped by adding dilute sulphuric acid and absorbance read at 630nm using Dynex Technologies ELISA microplate reader (Dynatech Laboratories, UK). Positive samples were defined as samples with optical density (OD) greater than 0.500.

Results

Cyclospora infections

On microscopic examination of faecal smears, *Cyclospora* oocysts were detected in 8 out of 10 male adults, 4 out of 7 female adults, 2 out of 3 male juveniles and 9 out of 13 female juveniles while the rest were *Cyclospora* negative and were used as controls.

Haematological Parameters Cyclospora negative AGM: The haematological parameters of *Cyclospor*a negative AGM studied at IPR were as presented in Table 1. The erythrocyte values were generally higher in the adults than in the juveniles. The red blood cells (x10^6/µl) varied between 5.2-6.69 for adult males and 3.7-5.14 for female juveniles. Similarly, the hemoglobin (g/dl) values had similar trend with a range between 10.9-14.2 for adult males and 8.7-12.7 female juveniles. The platelets (x 10^3/µl) varied from 110-612 with lowest values recorded in male juveniles and highest in female juveniles.

The white blood cells (x10^3/µl) varied among the study animals with a range of 2.9-8.7, with highest values recorded in male adults. The neutrophils ranged from 22-42% with higher values in male juveniles. Lymphocytes ranged from 52-78% with highest levels observed in male adults. Few monocytes and eosinophils were recorded in the study

animals while no basophils were detected. The total plasma protein ranged from 6.6-7-.5g/dl while plasma fibrinogen ranged from 0.2-0.4 g/dl (Table 1).

Cyclospora positive AGM: The hematological values for African green monkeys with-*Cyclospora* infections were analysed and were as presented in Table 2. Generally, the red blood cell counts decreased in all sex-age categories in *Cyclospora* positive animals. The red blood cells ($x10^6/\mu l$) decreased from 5.2-6.69 to 4.4-5.06 for adult males and from 3.7-5.14 to 2.8-5.6 for female juveniles. The hemoglobin (g/dl) values were increased froma range of 10.9-14.2 to 14.0-14.4 for adult males and from 8.7-12.7 to 12.0-12.2 for female juveniles.

The white blood cells ($x10^3/\mu l$) varied among the study animals. The female adults had an increase from 4.3-7.1 to 5.4-8.8 while the male adults had a decrease from 3.9-8.7 to 1.5-5.3. Among the juveniles, the males had an increase from 5.9-8.1 to 8.8-17.7 while the females showed a decrease from 3.3-7.3 to 1.4-3.5. The percent neutrophils increased in all sex-age categories with the highest increase in the male adults of 25-26% and lowest increase among the male juveniles of 17-21%. The per cent lymphocytes decreased in all sex-age categories with the highest drop observed among the male adults of 21-43% and lowest decline among the female adults of 7-2% (Table 1).

Cyclospora specific ELISA in Cercopithecus aethiops (African green monkeys) Cyclospora Infections Cyclospora negative AGM: Sera were probed for *Cyclospora* specific antibody responses using soluble *Cyclospora* proteins by ELISA. Negative *Cyclospora* responses were observed in thirteen of the study animals which corresponded to the animals that were *Cyclospora* negative on microscopy. Generally, the optical densities (OD) for male adults and the juveniles were similar with a mean value of 0.400 ± 0.010while the female adults had a mean value of 0.370 ± 0.010 (Figure 1).

Cyclospora positive AGM: Positive *Cyclospora* responses were observed in twenty of the study animals with higher values observed in juveniles than in adults (Figure 1). The female juveniles had the highest OD values with a mean value of 0.816 ± 0.100 and lowest among the male juveniles with a mean value of 0.646 ± 0.055 (Figure 1). The mean OD value for the adults was 0.776 and 0.738 for the males and females, respectively.

Red blood cells RBC, ($x10^6/\mu l$): Haemoglobin Hb, (g/dl): Haematocrit (%): Mean corpuscular volume MCV, (fl): Mean corpuscular haemoglobin MCH, ($\mu\mu g$): Mean corpuscular haemoglobin concentration MCHC, g/dl: White blood cells WBC, ($x10^3/\mu l$): Platelets Plt, ($x\ 10^3/\mu l$): Neutrophils Neu, (%): Lymphocytes Ly, (%): Monocytes Mo, (%): Eosinophils Eo, (%): Basophils Ba, (%): Total Plasma Protein TPP, (g/dl): Plasma Fibrinogen PF, (g/dl). §Data adapted from Kagira *et al* (2007) at 8 month adaptation period, ND- Not Determined.

Table 1: The Red Blood and White Blood Cell values of *Cyclospora* Negative African green monkeys studied at IPR, Nairobi from March 2008 to June 2009

	Adults		Juveniles		Normal Parameters Adult-Juveniles	
Blood Parameter	**Females Mean ±SD (Range)**	**Males Mean ±SD (Range)**	**Females Mean ±SD (Range)**	**Males Mean ±SD (Range)**	**Females Mean ±SD (Range)**	**Females Mean ±SD (Range)**
RBC	5.36±0.8 5.28-5.44	5.95±0.75 5.2-6.69	4.4±0.7 3.7-5.14	4.22±0.93 3.29-5.14	5.4± 0.5 4.8-6.2	5.2± 0.2 4.8-5.6
Hb	11.7±2.0 8.7-12.7	12.55±1.7 10.9-14.2	10.7±2.0 8.7-12.7	12.4±0.8 11.6-13.2	12.3± 1.2 10.5-15.2	11.8± 0.5 10.8-12.9
HCT	33.4±4.8 28.6-38.1	32.1±9.5 22.6-41.5	33.4±4.8 28.6-38.1	27.6±5.4 22.2-32.9	40.3± 4 33.4-49.3	39.7± 1.8 39.4-43.2
MCV	70.6±2.0 68.6-72.6	71.9±2.8 69.1-74.7	80.5±6.5 74.0-87.0	71.5±1.5 70.0-73.0	74.9± 3.9 67.8-79.1	75.8± 3.5 67.3-82
MCH	24.2±1.1 23.1-25.3	27.3±1.0 26.3-28.3	27.1±1.0 26.1-28.1	24.8±1.2 23.6-26.0	22.9± 1.1 21.1-24.4	22.4± 1 20.6-24.8
MCHC	25.8±5.3 20.5-31.1	30.0±1.1 28.9-31.1	32.6±1.7 31.1-34.4	30.6±0.5 30.0-31.1	30.7± 0.9 29.1-31.8	30.0± 0.8 28.9-31.6
Plt	528±125 403-653	489±127 362-616	586±65 521-650	215±105 110-320	386.7± 85 259-526	385.2± 80.8 321-519
WBC	5.7±1.4 4.3-7.1	6.3±2.4 2.9-8.7	5.3±2.0 3.3-7.3	7.0±1.1 5.9-8.1	7.5± 1.9 4.0-11.3	5.6± 1.1 4.3-6.9
Neu	35.5±5.5 30-41	32±10 22-42	36.5±4.5 32-41	40.5±0.5 40-41	3.6± 2.1 1.1-8.0	1.9± 1.1 0.6-3.7
Ly	61.5±8.5 53-70	67±11 56-78	62±5 58-67	52.5±0.5 52-53	3.4± 1.5 1.2-4.9	3.1± 0.6 2.5-4.0
Mo	0	0	1	0	0.6± 0.2 0.3-0.9	0.7± 0.3 0.3-1.2
Eo	0	2	2	3	ND	ND
Ba	0	0	0	0	ND	ND
TPP	7.5	7.4	6.6	6.6	ND	ND
PF	0.4	0.2	0.4	0.4	ND	ND

Table 2: Red Blood and White Blood Cell values of African green monkeys with *Cyclopsora* Infection studied at IPR, Nairobi from March 2008 to June 2009

	ANIMAL Sex/Age Class		*Cyclospora* Negative AGM	*Cyclospora* Positive AGM
Red blood cells X10^6	Female/ Adult		5.28-5.44	5.06-5.28
	Male /Adult		5.2- 6.99	4.4-5.06
	Female/ Juvenile		3.7-5.14	2.8-5.6
	Male/ Juvenile		3.29-5.14	3.1-4.5
Haemoglobin g/dl	Female/ Adult		8.7-12.7	9.8-10.3
	Male /Adult		10.9-14.2	14-14.4
	Female/ Juvenile		8.7-12.7	12.0-12.2
	Male/ Juvenile		11.6-13.2	10.1-11.0
White Blood cells X10^3	Female/ Adult		4.3-7.1	5.4-8.8
	Male /Adult		3.9-8.7	1.5-5.3
	Female/ Juvenile		3.3-7.3	1.4-3.5
	Male/ Juvenile		5.9-8.1	8.8-17.7
Neutrophils %	Female/ Adult		30-41	50-63
	Male /Adult		22-42	57-68
	Female/ Juvenile		32-41	55-67
	Male/ Juvenile		40-41	57-60
Lymphocytes %	Female/ Adult		53-70	46-68
	Male /Adult		56-78	25-35
	Female/ Juvenile		58-67	48-52
	Male/ Juvenile		52-53	38-42

Discussion

Cyclospora infections were recorded in all sex-age categories with an overall prevalence of 70% in the animals studied. The finding was in general agreement with prevalence reported by other authors (Eberhard *et al.*, 2001). The slightly higher value reported in this study maybe as result of few animals investigated and a large number being juveniles, 16 out of 33 while Eberhard *et al.* (2001) reported *Cyclospora* infections with a prevalence of 25-65% and mean prevalence of 35%. The finding of no *Cyclospora* parasites in faecal samples of the two colonies bred was significant in quality assurance of the animals in the established colony at IPR.

The general trends of the haematology values of the *Cyclospora* negative AGM were that the erythrocyte values higher in the male adults and lower in the female juveniles while the white blood cell counts varied among the different study animals with the highest values recorded among the male adults. The haematological values recorded in this study for red and white blood cells are in general agreement with published data as reported by other authors (Schalm *et al.*, 1975; Kagira *et al.*, 2007). This study provides additional

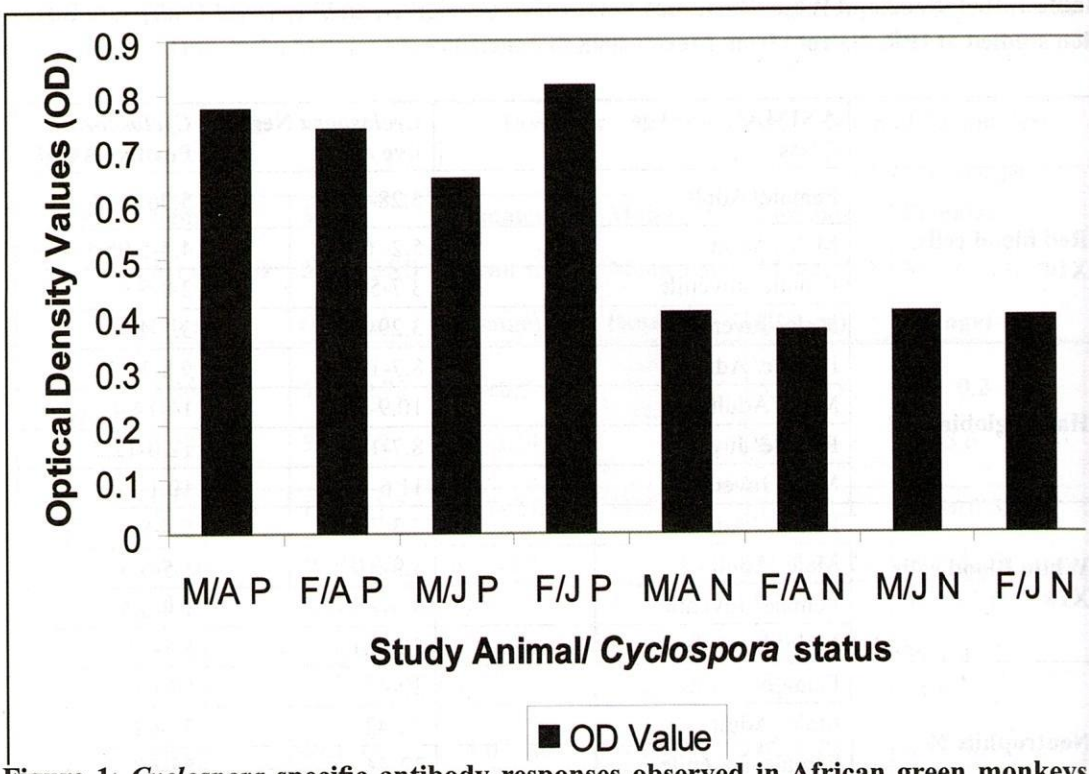

Figure 1: ***Cyclospora*** **specific antibody responses observed in African green monkeys with*Cyclospora* infections at IPR in June 2009. "M" for male, "F" for female, "A" for adult, "J" for juvenile, "P" for *Cyclospora* positive and "N" for *Cyclospora* negative.**

data on differential cell counts for eosinophils, basophils and total plasma proteins and plasma fibrinogens in Kenyan AGM which had not been previously reported by Kagira *et al.* (2007) but is in agreement with published data by Schalm *et al.* (1975).

Age- and sex-related haematology values have been described in vervet monkeys by Sato *et al.* (2005), who reported that male vervets tended to have higher erythrocyte and haematocrit values than females. The higher values in adult males can be attributed to production of male sex hormone and higher muscular mass. The haematological changes in vervet monkeys during eight months adaptation to captivity with most haematological parameters becoming stable after 4 months have been described (Kagira *et al.*, 2007). However, published data shows wide animal variations in haematological parameters in vervet monkeys (Schalm *et al.*, 1975) hence the necessity of establishing baseline haematological data in the current study.

The trends of the haematology values of the *Cyclospora* positive AGM werea general decline in the red blood cell values observed in all sex-age categories while the white blood cell counts were increased in female adults and male juveniles but decreased in the other sex-age categories. The decline in red blood cells which may have been caused by a depletion of existing cells or low production of cellsmay be an effect of *Cyclospora* infec-

tion. Consequently, the haemoglobin values were increased marginally in all the study animals.

The total white blood counts (WBC) in the *Cyclospora* infected AGM were higher in adult females than in other animals. The increase in WBC was closely associated with an increase in neutrophil percent. This increase in neutrophils would indicate a positive to*Cyclospora* infection. A decline in lymphocyte percent was observed in all the Cyclospora positive AGM. This decline in lymphocyte percent would indicate depletion in this cell population either by destruction of existing cells or lack of proliferation of the cells caused by the *Cyclospora* infection.

Cyclospora specific antibody responses with high optical density values were observed in the *Cyclospora* positive animals. These observations were significant in that the antibody responses were an indication of an active immune system that was mounting defense to invading micro-organisms. Low *Cyclospora* antibody responses were observed in the negative controls. These responses were as anticipated and display the body's immune response to infection.

In conclusion, this study has demonstrated that African green monkeys with *Cyclospora* infections lead to both haematological and serological changes in the animal body that can be assayed.

Acknowledgements

The authors would like to acknowledge the technical assistance given by staff in Animal Resource and Tropical Infectious Diseases Departments of Institute of Primate Research, Nairobi.

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